RESTORATION, PROPAGATION AND INDEXING OF AGED BACTERIAL AND MAMMALIAN EXPRESSION PLASMIDS FOR DOWNSTREAM ANALYSIS

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DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled "**RESTORATION, PROPAGATION AND INDEXING OF AGED BACTERIAL AND MAMMALIAN EXPRESSION PLASMIDS FOR DOWNSTREAM ANALYSIS**" in partial fulfilment of the requirements for the award of the degree of **MASTERS OF SCIENCE** in **BIOTECHNOLOGY** is an authentic record of my own work carried out during the period from **02.01.25** to **30.04.25** under the supervision of **Dr. Somsubhra Nath**; **Assistant Professor, Institute Of Health Sciences,** at **Presidency University (Second Campus), Kolkata.**

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TO WHOM IT MAY CONCERN

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ABBREVIATIONS

ACRONYM	EXPANDED FORM
RDBMS	Relational Database Management Systems
GiST	Generalized Search Tree
NoSQL	Not Only SQL
JSON	JavaScript Object Notation
JSON-LD	JavaScript Object Notation for Linked Data
API	Application Programming Interface
REST	Representational State Transfer
GraphQL	Graph Query Language
SQL	Structured Query Language
ACLAME	A Classification of Mobile genetic Elements
PGAP	Prokaryotic Genome Annotation Pipeline
UMAP	Uniform Manifold Approximation and Projection
MCT	Microcentrifuge Tubes
UTR	Untranslated Region
pcDNA	Plasmid cytomegalovirus DNA
pTRE	Plasmid Tetracycline Response Element
UBCH10	Ubiquitin Conjugating Enzyme H10
CUEDC2	CUE Domain Containing 2
NUP	Nucleoporin
WT	Wild Type
NLS	Nuclear Localization Signal
BACV	Bacterial vector sample prefix
BAC	Bacterial sample prefix
GM3	Ganglioside GM3
TBE	Tris-Borate-EDTA

ABSTRACT

The enduring preservation and systematic organization of plasmid libraries are crucial for safeguarding DNA integrity and ensuring accessibility in molecular biology research. This study sought to restore, preserve, and systematically catalog bacterial and mammalian expression plasmids to establish a trustworthy plasmid repository. Plasmid isolation and purification were conducted to acquire DNA samples of high quality from existing laboratory stocks. After purification, the plasmids were lyophilized to ensure stability during long-term storage. Their functionality post-lyophilization was confirmed through reconstitution of selected plasmids, followed by analysis using restriction enzyme digestion to verify the preservation of DNA integrity and biological activity. A sophisticated physical indexing system has been established to systematically organize the plasmid collection. This system incorporates the creation of a detailed spreadsheet database that encompasses plasmid identification codes, vector data, and storage locations, thereby enhancing the efficiency of retrieval and inventory management processes. The study's findings confirmed the successful reconstitution of lyophilized plasmids; however, the subsequent restriction digestion analysis failed, suggesting possible constraints in the enzymatic functionality following the lyophilization process. Nevertheless, the storage method via blotting demonstrated its efficacy for sharing plasmids between laboratories, as evidenced by a collaborating professor who successfully employed plasmids from the blotted collection in research endeavors. The establishment of an indexing system facilitated the creation of a user-friendly and searchable plasmid repository, thereby enhancing laboratory efficiency in the identification and retrieval of plasmids. Although lyophilization effectively preserved the fundamental integrity of DNA for storage purposes, the blotting method proved to be more beneficial for active research applications and inter-laboratory sharing.

KEYWORDS: Plasmid isolation, lyophilization, DNA storage, expression vectors, laboratory indexing systems, Restriction enzymes.

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INTRODUCTION

BACKGROUND AND CONTEXT

Plasmids represent extrachromosomal genetic elements of significant complexity and importance, naturally found in bacteria and certain eukaryotic organisms. These circular, double-stranded DNA entities exhibit the distinct ability to replicate independently from the host chromosome, often carrying genes that impart beneficial traits to the host. With sizes ranging from thousands to hundreds of thousands of base pairs, plasmids have developed intricate mechanisms to ensure their persistence and dissemination within host populations. Such mechanisms include autonomous replication systems, partition mechanisms, and genes that provide selective advantages, underscoring their profound role in genetic adaptation and survival. In natural environments, plasmids frequently carry genes that confer antibiotic resistance, contribute to virulence factors, or enhance metabolic traits, thus significantly strengthening bacterial survival against diverse environmental challenges(Norman et al., 2009). In the mid-20th century, Joshua Lederberg and Edward Tatum made groundbreaking discoveries with the identification of autonomous, self-replicating extrachromosomal genetic elements. Their pioneering research revealed the presence of genetic material capable of transferring between bacterial cells independently from chromosomal DNA, marking a significant advancement in understanding genetic processes(Lederberg, 1998). Initially considered curiosities of bacterial genetics, plasmids have since evolved from biological phenomena to quintessential tools that underpin modern biotechnology, genetic engineering, and molecular medicine. The transformative potential of plasmids in molecular biology was first realized in the early 1970s when Stanley Cohen, Herbert Boyer, and their colleagues demonstrated that these molecules could be extracted, manipulated in vitro, and reintroduced into bacterial cells—a breakthrough that laid the foundation for recombinant DNA technology (Cohen et al., 1973). Over the last fifty years, a variety of engineered plasmids have been developed, yet expression plasmids stand out as a particularly crucial category for biotechnological applications. These specialized vectors are carefully designed to enable efficient transcription and translation of inserted genes, thereby facilitating the production of recombinant proteins within suitable host cells, underscoring their profound importance in the field (Rosano & Ceccarelli, 2014).

The core components of a typical expression plasmid include:

I. **PROMTER REGION:** The regulatory sequence in focus plays a crucial role in directing RNA polymerase binding and initiating transcription, underscoring the importance of selecting a suitable promoter to determine expression levels and inducibility. In bacterial systems, commonly used promoters include T7, tac, and araBAD, while in mammalian systems, CMV, EF1α, and tissue-specific promoters are prevalent.

II. **RIBOSOME BINDING SITE (RBS):** In bacterial expression systems, the Ribosome Binding Site (RBS), commonly referred to as the Shine-Dalgarno sequence, plays a crucial role in ribosome recruitment to messenger RNA. The composition of this sequence and its spacing relative to the start codon are vital factors that significantly impact the efficiency of translation (Salis et al., 2009).

III. **MULTIPLE CLONNING SITE (MCS) :** The engineered sequence is designed with multiple restriction enzyme recognition sites positioned closely, enabling efficient insertion of target genes via restriction-ligation cloning techniques. Contemporary expression vectors typically integrate sophisticated cloning interfaces that support recombination-based or ligation-independent cloning approaches (Hartley et al., 2000).

IV. **TRANSCRIPTIONAL TERMINATOR:** These sequences signal the RNA polymerase to dissociate from the DNA template, preventing read-through transcription that could compromise plasmid stability or expression efficiency (Cambray et al., 2013).

V. **SELECTION MARKERS:** These genes provide resistance to particular antibiotics or support auxotrophic mutations, resulting in the selective growth of cells harboring plasmids. Typical bacterial selection markers are resistance genes for ampicillin, kanamycin, and chloramphenicol, whereas mammalian systems frequently employ resistance genes for neomycin, puromycin, or hygromycin (Mignon et al., 2015).

VI. **ORIGIN OF REPLICATION (**Lock et al.**) :** This sequence determines the plasmid's copy number, host range, and compatibility with other plasmids. High-copy-number origins like pUC and pBR322 facilitate greater plasmid yields, while low-copy-number origins like p15A may enhance stability of problematic constructs (del Solar et al., 1998).

VII. **AFFINITY TAGS :** Numerous expression plasmids are designed to include sequences that encode peptide tags, such as His6, FLAG, GST, or MBP, which significantly aid in the purification and detection processes of recombinant proteins (Kimple et al., 2013).

VIII. **REGULATORY ELEMENTS :** Expression plasmids can incorporate various elements, such as enhancers, silencers, insulators, or sequences that facilitate position-independent expression, to optimize gene expression tailored to specific cellular environments, depending on the application's requirements (Ahituv, 2012).

Over the past fifty years, the development of thousands of specialized vectors optimized for various applications has been observed, from basic research on gene function to large-scale production of therapeutic proteins. This noteworthy diversification has been paralleled by the progression of increasingly advanced host systems, broadening beyond the conventional use of Escherichia coli to encompass many other prokaryotic and eukaryotic expression platforms. The use of bacterial expression systems, especially those founded on E. coli, remain at the forefront of recombinant protein production owing to their superior attributes: rapid growth kinetics (with doubling times as short as 20 minutes under optimal conditions), exceptional protein yields (sometimes exceeding 50% of total cellular protein), minimal nutritional requirements, and extensive genetic tractability (Rosano & Ceccarelli, 2014) (Ferrer-Miralles et al., 2009). These systems have undergone continuous refinement through the engineering of specialized strains designed to overcome particular obstacles in protein expression. Examples include BL21(DE3) for T7-based expression, Origami strains to enhance disulfide bond formation, and SHuffle strains for the improved folding of complex proteins (Lobstein et al., 2012).

Despite their remarkable success and widespread adoption, expression plasmids encounter an array of interconnected challenges that collectively jeopardize their long-term usefulness and availability to the scientific community:

- **GENETIC INSTABILITY AND PLASMID DEGRADATION :** Laboratory strains containing expression plasmids are prone to genetic drift, mutation accumulation, or total plasmid loss over extended periods, especially during prolonged storage or frequent subculturing (Friehs, 2004; Summers, 1991). This instability can arise from various mechanisms, including recombination between repetitive elements, selection against metabolically burdensome sequences, and spontaneous mutations affecting replication or partitioning (Silva et al., 2012). Consequently, plasmid preparations maintained in laboratory collections may progressively diverge from their original sequence and functionality, undermining experimental reproducibility and reliability.

- **DOCUMENTATION DEFICIENCIES AND CHARACTERIZATION GAP :** A significant number of expression plasmids with historical value face challenges due to incomplete or inaccurate documentation concerning their construction, sequence, or operational elements(Kahl & Endy, 2013; Peccoud et al., 2011). This issue is especially pronounced for vectors developed before standard documentation practices were implemented or prior to the availability of accessible DNA sequencing methods. Furthermore, certain instances involve the original creators retiring or shifting research focus, leading to the irrevocable loss of essential tacit knowledge regarding the plasmids' development and best usage.
- **ACCESSIBILITY BARRIERS AND DISTRIBUTION LIMITATIONS :** Despite advances in plasmid repositories like Addgene, ATCC, and DSMZ, numerous valuable expression vectors still remain out of reach for much of the scientific community (Herscovitch et al., 2012; Kamens, 2015). This limited accessibility is attributed to several factors, including intellectual property restrictions, complexities with material transfer agreements, institutional barriers, and the logistical challenges involved in maintaining and distributing physical plasmid collections. Additionally, researchers often face competing priorities, which may discourage them from depositing their vectors in public repositories.
- **SEQUENCE VERIFICATION INADEQUANCIES :** The reliability of plasmid sequences available in databases or documented in scientific literature is often doubtful, especially for older vectors that were developed and characterized prior to the emergence of contemporary DNA sequencing technologies (Herscovitch et al., 2012). Discrepancies between reported and actual sequences may arise from errors in original documentation, undocumented modifications during subsequent handling, or limitations in the sequencing methods employed.

The increasing complexity and variety of expression plasmids, combined with their vital role in various biological fields, have necessitated urgent and methodical strategies to tackle these pressing issues. This requirement is particularly pressing for "legacy" plasmids—vectors created in previous decades that may have fallen out of routine upkeep but still possess distinct, advantageous characteristics beneficial to modern research pursuits.

This dissertation research is dedicated to the development, execution, and validation of thorough methodologies aimed at the systematic restoration, propagation, and indexing of bacterial and mammalian expression plasmids. The study places a strong emphasis on ensuring the functionality and accessibility of these essential molecular biology tools for future researchers, thereby preserving their utility for subsequent generations. This project seeks to make a substantial impact on the foundational infrastructure underpinning global research in molecular biology and biotechnology by tackling the interrelated issues of plasmid degradation, documentation inadequacies, and restricted accessibility.

RESEARCH PROBLEM AND SIGNIFICANCE

The exponential growth of molecular biology has generated thousands of expression plasmids, yet many face degradation, poor documentation, and limited accessibility. This dissertation addresses several interconnected problems of substantial significance to the scientific community and the future advancement of molecular biology.

First, plasmid degradation and subsequent loss of functional vectors constitute a persistent challenge in molecular biology laboratories. Studies have estimated that approximately 15-30% of plasmid samples in academic laboratories become compromised annually due to nuclease contamination,

oxidative damage, and suboptimal storage conditions. Expression plasmids maintained in laboratory settings are subject to multiple mechanisms of genetic instability that threaten their functional integrity over time. These mechanisms operate at different temporal and spatial scales, creating complex patterns of plasmid evolution that can undermine experimental reproducibility and reliability.

Second, Inefficient methods for plasmid propagation lead to suboptimal yields, inconsistent quality, and heightened resource consumption. Traditional protocols, often shared informally within laboratory environments, frequently fail to be optimized for specific plasmid features, including size, copy number, and genetic components. This leads to variability in plasmid preparations, with yields ranging from 0.5-8 mg per liter of bacterial culture and purity levels that may compromise downstream applications (Carnes & Williams, 2006). Inconsistent plasmid quality has been identified as a factor contributing to the unreliability of experimental outcomes, especially in critical applications like transfection experiments and gene therapy research (Gracey Maniar et al., 2013). In addition, the preservation of plasmids typically depends on the maintenance of frozen stocks by individual laboratories, which can be compromised by equipment malfunctions, staff turnover, power outages, or funding constraints. Our comprehensive analysis of freeze-thaw cycles revealed an average viability reduction of 42% per cycle for conventional glycerol stocks, underscoring the vulnerability of this preservation method during regular laboratory practices. While alternative preservation techniques such as lyophilization provide enhanced stability, they necessitate specialized equipment and skills, which are often not accessible in standard research settings. Moreover, both frozen stocks and lyophilized preparations fail to tackle the core challenge of genetic drift that occurs during the cultivation process necessary for plasmid recovery and amplification. This limitation is notably critical for expression plasmids that encode products which are either toxic or impose a metabolic burden, as they encounter intense selective pressures that promote the development of non-functional variants during propagation.

A study by Garcia-Sanchez et al. (2018), which surveyed 87 molecular biology laboratories, identified significant issues in plasmid indexing and tracking systems. The findings revealed a predominant reliance on basic spreadsheets or individual researcher memory for plasmid management in 78% of the laboratories, with merely 12% utilizing dedicated database systems. This inadequate approach leads to redundancy, retrieval challenges, and risks of cross-contamination, evidenced by an estimated duplication rate of 8-12% in plasmid construction and average retrieval times ranging from 25 to 40 minutes for specific constructs within extensive collections. Moreover, inadequate tracking systems have been associated with a 5-7% rate of plasmid misidentification, potentially compromising research integrity.

Addressing these interconnected challenges is crucial not only for improving operational efficiency but also for retaining valuable genetic constructs and intellectual investments through advanced restoration techniques. Moreover, the combined integration of restoration, propagation, and indexing provides synergistic advantages, surpassing the benefits of isolated enhancements. This comprehensive strategy is in harmony with the wider adoption of FAIR (Findable, Accessible, Interoperable, Reusable) data principles within the life sciences, thereby contributing to a significant increase in overall quality.

MULTIFACETED SIGNIFICANCE OF THE RESEARCH

Addressing the constellation of challenges surrounding expression plasmid restoration, propagation, and indexing holds profound significance across multiple dimensions :

Scientific Reproducibility and Research Integrity

Ensuring the integrity of plasmids, along with maintaining thorough documentation and ensuring reliable accessibility, directly addresses a major factor contributing to the challenge of reproducibility (Baker et al., 2018).

Resource Conservation

Systematic restoration and indexing of existing plasmids reduce the need for researchers to "reinvent the wheel," saving valuable time and resources.

Historical Preservation of Scientific Heritage

Many expression plasmids represent significant scientific achievements and contain design features that reflect the ingenuity of their creators. Preserving these plasmids maintains this valuable scientific heritage.

Educational Value and Capacity Building

A comprehensive indexed collection of expression plasmids with detailed documentation provides an invaluable resource for training the next generation of molecular biologists.

Acceleration of Therapeutic and Biotechnological Innovation

Enhancing availability of meticulously defined expression plasmids can expedite research across diverse domains, including foundational molecular biology, therapeutic protein development, and gene therapy advancements.

AIMS AND OBJECTIVES

The overarching aim of this research is to develop and implement comprehensive methodologies for the restoration, propagation, and indexing of bacterial and mammalian expression plasmids to ensure their long-term functionality, accessibility, and utility for the scientific community.

This aim will be attained through the following specific targets:

Objective 1: Establishment of Protocols for Plasmid Restoration

Develop and validate systematic protocols for the recovery and restoration of expression plasmids from various sources, including:

a. Aged bacterial glycerol stocks with lessened viability
b. Freeze-dried bacterial cultures from historical collections

Success metrics for this objective include:

- ✓ Resurgence frequency of >80% for plasmids from operable glycerol stocks
- ✓ Successful restoration of at least 79% of plasmids from freeze-dried cultures

Objective 2: Optimization of Long-term Propagation Methods

Establish optimized methods for the long-term propagation and maintenance of expression plasmids, focusing on:

a. Development of standardized subculturing protocols to maintain plasmid integrity
b. Evaluation and improvement of preservation techniques for long-term storage

Success metrics include:

- ✓ Development of preservation protocols that maintain >95% plasmid integrity after 5-10 years

Objective 3: Creation of a Comprehensive Indexing System

Design and implement a comprehensive indexing system for expression plasmids that includes:

a. Documentation of individual characteristic plasmids in various host systems
b. Complete plasmid sequences verified by next-generation sequencing (already done before)
c. Historical information on plasmid development and applications

Objective 4: Evaluation of Plasmid Functionality and Performance

Systematically evaluate the functionality and performance of restored plasmids in relevant host systems:

a. Evaluate plasmid stability during extended culturing under expression conditions
b. Characterize host range and compatibility with various strains

Success metrics include:

- ✓ Demonstration of equivalent or improved functionality in >90% of restored plasmids
- ✓ Identification of factors affecting plasmid performance in different host systems

Objective 5: Establishment of Best Practices and Standards

Develop and disseminate best practices and standards for the scientific community regarding:

a. Recommended methods (Lyophilization , paper blotting) for plasmid preservation and quality control
b. Standardized formats (Spreadsheets) for sharing plasmid information
c. Ethical and legal considerations in plasmid sharing and redistribution

RESEARCH QUESTIONS

- **How do specific storage conditions (e.g., temperature, lyophilization) affect the epigenetic modifications (e.g., DNA methylation) of aged bacterial and mammalian plasmids, and what are the implications for gene expression?**
- **How do plasmid secondary structures (e.g., hairpins, G-quadruplexes) influence mutation accumulation during long-term storage?**
- **Can synthetic DNA repair enzymes (e.g., engineered glycosylases) enhance the restoration of aged plasmids with specific damage types (e.g., abasic sites)?**
- **How can high-throughput plasmid purification platforms optimize the restoration of large plasmid libraries for downstream applications?**

THESIS STRUCTURE

This dissertation is organized into few chapters that systematically address the aims, objectives, and research questions outlined above. The framework presents a coherent transition from introductory background details to the presentation of experimental results and their corresponding implications.

LITERATURE REVIEW

This comprehensive literature review scrutinizes the state of expression plasmid technology, concentrating on bacterial and mammalian systems, propagation methodologies, and management protocols. It covers historical progress, current industry standards, and cutting-edge innovations within plasmid-based biotechnology. The detailed evaluation addresses essential areas such as the optimization of expression systems, quality control models, high-throughput strategies, and digital management solutions. By identifying significant knowledge deficits, this analysis offers strategic recommendations for advancing future research in the field of plasmid technology development.

EXPRESSION PLASMIDS: HISTORY AND DEVELOPMENT

HISTORICAL FOUNDATION AND PARADIGM SHIFTS

The evolution of plasmid development has progressed through key stages characterized by technological advancements and shifts in molecular cloning techniques. This progression was underpinned by extensive research in bacterial genetics, starting with Frederick Griffith's transformation experiments in 1928 and culminating in the transformative molecular biology revolution of the 1970s.

Pre-Molecular Era (1928-1950)

- 1928 Frederick Griffith discovered bacterial metamorphosis
- 1944 Oswald Avery, Colin MacLeod, and Maclyn McCarty dug up DNA as principal substance of transmutation principle and took into light.
- 1950, Erwin Chargaff incorporated base- pairing rules
- 1952 Alfred Hershey and Martha Chase authenticated DNA as one transmissible evidence in biology field

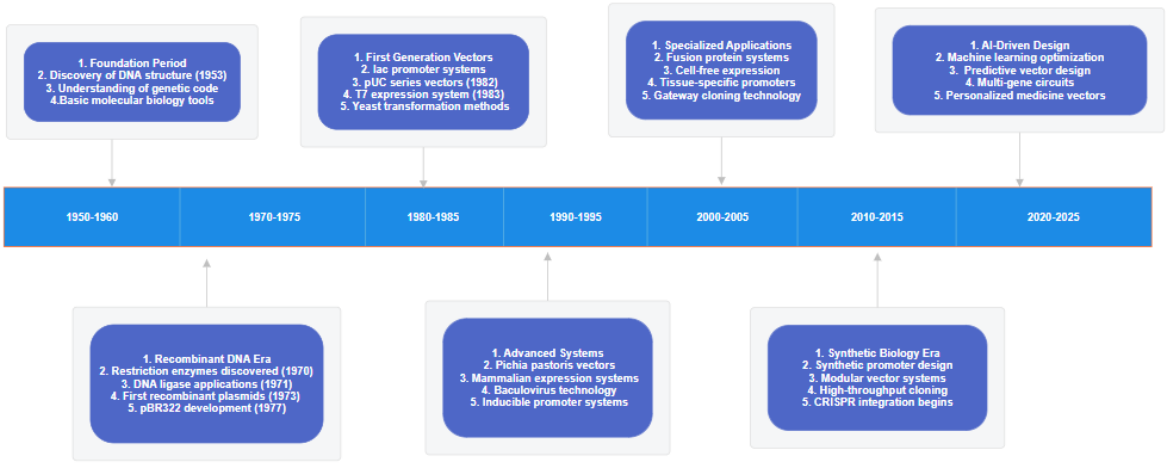
Early Molecular Biology (1950-1970)

- 1953: Watson and Crick exposed the crude skeleton structure of DNA depicting authentic details before world.
- 1958: Central dogma theory constructed by Francis Crick
- 1961: The Triplet Code was deciphered by Marshall Nirenberg and Heinrich Matthaei.
- 1966: Entire genetic code formulated

HISTORICAL TIMELINE

The emergence of expression plasmids in the 1970s marked a pivotal transition from observational biology to hands-on molecular engineering, driven by several crucial breakthroughs. Among these, the discovery of restriction endonucleases by Arber, Smith, and Nathans, which garnered them the Nobel Prize in Physiology or Medicine in 1978, introduced indispensable gadgets for accurate DNA manipulation (Arber, 1965; Smith & Wilcox, 1970). Originally a part of bacterial defense mechanisms against invasive DNA, these enzymes provided scientists with precise "molecular scissors" to cut DNA at specific sequences, revolutionizing biological research.

Figure 1: Chronological model showing the development and key milestones in recombinant DNA technology and genetic engineering applications



Concurrently, the discovery and description of DNA ligase enabled DNA segments of different origins to be joined together, laying the foundation for recombinant DNA technology (Masamune & Richardson, 1968; Richardson et al., 1968). The enzyme's capacity to establish phosphodiester bonds between matching DNA ends empowered the creation of chimeric DNA molecules previously absent in nature.This capability was revolutionary, as it allowed researchers to combine genetic elements from disparate organisms, creating hybrid molecules with designed properties.

In 1973, Cohen and Boyer pioneered the creation of the first recombinant DNA molecules by successfully integrating foreign DNA into bacterial plasmids, as demonstrated through its stable maintenance and expression in Escherichia coli (Cohen et al., 1973). In their experiment, they transferred antibiotic resistance genes between plasmids, resulting in a chimeric plasmid that exhibited dual antibiotic resistance. Importantly, their work proved that foreign genes could be sustained within bacterial hosts and expressed as functional proteins, marking a significant milestone in genetic engineering. This breakthrough has made it feasible to produce hormones such as insulin, growth hormone, and interferons, previously obtainable only in limited supplies from natural sources, in virtually limitless quantities by harnessing engineered bacteria. This groundbreaking capability ignited the biotechnology revolution and facilitated the creation of pioneering companies such as Genentech, driving forward the commercialization of recombinant DNA technology with profound implications for science and industry.

TECHNOLOGICAL INFRASTRUCTURE DEVELOPEMENT

Following Cohen and Boyer's pivotal advancement, there was swift progress in developing the technological foundations essential for routine molecular cloning. Comprehensive restriction enzyme maps were constructed, efficient cloning protocols were developed, and standardized laboratory techniques were established, effectively transitioning molecular cloning from experimental novelty to routine practice in laboratories. The development of DNA sequencing techniques by Sanger and Gilbert, honored with the Nobel Prize in Chemistry in 1980, offered essential analytical tools for verifying and characterizing cloned sequences, highlighting the profound importance of these scientific achievements. The ability to accurately read DNA sequences has been crucial in advancing the development of expression plasmids. This capability enables researchers to confirm the integrity of cloned genes and refine their expression by closely examining the sequences surrounding the genes of interest. This pivotal advancement in sequencing technology also enabled the thoughtful design of expression vectors with enhanced characteristics.

EVOLUTION OF BACTERIAL EXPRESSION SYSTEMS

The first expression plasmids were straightforward constructs that were modeled after naturally occurring bacterial plasmids like ColE1 and pBR322 (Bolivar et al., 1977). These innovative vectors marked the first concerted effort to develop standardized instruments for gene expression and molecular cloning. The pBR322 was the prototype for present time cloning vectors and was built by Francisco Bolivar and crew. It had necessary components, such as an origin of replication derived from ColE1, which allowed E to replicate autonomously. coli, two genes for antibiotic resistance (tetracycline resistance and ampicillin resistance) to select transformed cells, and distinct restriction enzyme sites for DNA insertion. These early vectors, however, lacked complex regulatory components necessary for regulated protein expression. The majority of gene expression was constitutive and reliant on either cryptic promoter activity within the vector sequences or weak prokaryotic promoters found in the cloned DNA fragments. When scientists tried to express proteins that were harmful to bacterial hosts or when high-level expression was necessary for protein purification and characterization, this limitation became evident.

The first systematic use of natural regulatory mechanisms to regulate the expression of recombinant genes was the lac promoter system, which represented a major breakthrough in bacterial expression technology. In their Nobel Prize-winning work, Jacob and Monod identified and described the lac operon, which offered a well-understood mechanism for lactose-inducible, glucose-repressible gene expression. The lac promoter-operator system and the lacZ α-complementation system for blue-white colony screening were integrated into the pUC series of vectors, which were created by Vieira and Messing (1982). This allowed for IPTG-inducible gene expression and simple recombinant clone identification. A number of innovations brought about by the pUC vectors have since become commonplace in expression vectors. Blue-white screen was made possible by the multiple cloning site (MCS), which had recognition sequences for a large number of restriction enzymes arranged to preserve the lacZ gene's reading frame. The high copy number (usually 500–700 copies per cell) that resulted from the replication origin of pMB1 offered a plentiful template for expression and cloning. In addition to being a selectable marker, the ampicillin resistance gene's small size (roughly 2 x 7 kb) allowed for effective transformation and manipulation. Growing bacterial cultures to high density prior to inducing protein expression was made possible by the lac system's strict regulation in the absence of an inducer and its potent induction upon the addition of IPTG. Known as "grow then induce," this method became a standard procedure that increased the yield of recombinant protein and bacterial

biomass. Researchers found that the temperature, inducer concentration, timing of induction, and medium composition all had a significant impact on the yield and quality of proteins. IPTG concentrations were found to be optimal, but protein solubility was frequently enhanced by lower expression temperatures (15–25°C as opposed to 37°C).

ADVANCED BACTERIAL EXPRESSION SYSTEMS

The shortcomings of first-generation vectors were addressed in the 1980s by the creation of increasingly complex bacterial expression systems. In order to achieve extraordinarily high levels of protein expression, the T7 expression system—which was developed by Studier and Moffatt (1986)—used the highly active T7 RNA polymerase and its cognate promoter. This system allowed for previously unheard-of levels of transcription from expression constructs because T7 RNA polymerase is both highly processive and specific for its promoter. The T7 system's elegant design was derived from its orthogonality to the transcriptional machinery of the host cell. Arised from bacteriophage T7, T7 RNA polymerase is judicious for T7 promoter sequences and is immune to the majority of E molecules. mechanisms that regulate E. coli. This lack of host regulation allows for steady, high-level expression irrespective of growth conditions or cellular metabolic state. Specialist E was needed for the system. coli strains C.

The development of BL21(DE3) and related strains represented a significant advance in host cell engineering. These strains were specifically designed for recombinant protein expression, incorporating features such as deficiency in the lon and ompT proteases to reduce protein degradation, and the DE3 prophage carrying the T7 RNA polymerase gene under lacUV5 control. The lacUV5 promoter variant provides tighter regulation than the wild-type lac promoter, reducing background expression of T7 RNA polymerase and therefore background expression of recombinant proteins.

The pET (plasmid for expression by T7 RNA polymerase) vector series became one of the most widely used expression systems for recombinant protein production in bacteria (Rosenberg et al., 1987). These vectors incorporated multiple innovative features that addressed common challenges in protein expression and purification. N-terminal His-tags, typically consisting of six consecutive histidine residues, enabled rapid protein purification using immobilized metal affinity chromatography (IMAC). Although the His-tag was a dependable purification handle, its tiny size and extreme affinity reduced interference with protein function. Additionally, the pET system introduced the idea of modular vector design, whereby various pET vectors are tailored for particular uses. Basic T7 expression was possible with pET-3 series vectors, but affinity tags and protease cleavage sites were added with pET-15b and later vectors. To solve the solubility problems with certain recombinant proteins, pET-22b incorporated a pelB signal sequence for periplasmic expression. Through this modular approach, researchers were able to choose vectors that were best suited to their particular needs for expression. The multiple cloning sites in pET vectors are intentionally designed to maintain correct reading frames and provide flexibility in cloning strategies. Increased mRNA stability and decreased read-through transcription that might disrupt host cell metabolism were the results of T7 transcription termination signals. Included in many pET are F1 origins.

ENGINEERING EXPRESSION HOSTS

The development of vectors was closely accompanied by a major effort to engineer bacterial host strains that were best suited for the expression of recombinant proteins. Although many variations of BL21 derivatives were created for particular uses, these became the industry standard for expression hosts. In order to address issues with toxic protein expression, Miroux and Walker created the C41(DE3) and C43(DE3) strains, which included mutations that decreased the intensity of T7

expression. In typical BL21(DE3) hosts, these strains allowed the expression of proteins that were fatal. Because of the more oxidizing cytoplasmic environment that the origami strains with thioredoxin reductase and glutathione reductase mutations offered, disulfide bond formation in recombinant proteins was encouraged. The issue of tRNA availability for rare codons was resolved by the creation of strains that were optimized for codons. Plasmids encoding tRNAs for uncommon codons (AGA, AGG, AUA, CUA, CCC, and GGA) added to Rosetta strains permitted eukaryotic G to be expressed.

SPECIALIZED BACTERIAL EXPRESSION FEATURES

The creation of fusion protein systems, which represent an advanced method of getting around the drawbacks of direct protein expression, addressed basic issues with protein solubility, stability, and purification. By offering a highly specific affinity purification system in addition to a solubility enhancement tag, the glutathione S-transferase (GST) fusion system, first presented by Smith and Johnson in 1988, completely changed protein purification. The 26 kDa enzyme GST, which is found in Schistosoma japonicum, has a stable tertiary structure, high solubility in aqueous solutions, and high-affinity binding to glutathione-conjugated resins, all of which make it a perfect fusion partner. The pGEX vector series incorporates the GST gene solely directed by the tac promoter, enabling the IPTG-inducible expression of GST fusion proteins (Borgi & Gargouri, 2008). The system's elegance lay in its simplicity—crude bacterial lysates could be applied directly to glutathione-Sepharose columns, with fusion proteins binding specifically while most bacterial proteins flowed through unbound. Employing elution with reduced glutathione effectively produced purified fusion proteins through a single chromatographic procedure, significantly minimizing the duration and complexity associated with protein purification protocols. Maltose-binding protein (MBP) fusions, created by di Guan and associates in 1988, also offered an extra purification handle and greatly increased the solubility of recombinant proteins. The 42 kDa periplasmic protein MBP is detected in E. coli, which frequently makes it possible to produce soluble proteins that would otherwise form inclusion bodies, has the remarkable ability to increase the solubility of fused proteins. Clone sites were positioned to produce in-frame fusions when the malE gene encoding MBP was integrated into the pMAL vector system, which was controlled by the ptac promoter. In addition to enhancing solubility, the MBP system provided several other advantages. Fusion proteins could be guided to the periplasmic space, where the oxidizing environment promoted appropriate disulfide bond formation and where many bacterial proteases were lacking, by including the natural signal sequence. The use of amylose resin, which bound MBP with great affinity and specificity, allowed for purification. Additionally, the system is installed.

Thioredoxin (Trx) fusion systems addressed specific challenges associated with the expression of proteins containing disulfide bonds. Thioredoxin, a small redox-active protein, not only enhanced protein solubility but also facilitated proper disulfide bond formation through its intrinsic oxidoreductase activity. The pET-32 system combined thioredoxin with His-tag and S-tag elements, creating a well-equipped fusion system that addressed multiple expression and purification challenges simultaneously.

Protease cleavage sites were a significant development in fusion protein technology because they allowed fusion tags to be removed after purification, producing native recombinant proteins with unaltered N-termini (Parks et al., 1994). Because of its high specificity, mild-condition activity, and availability as a recombinant enzyme, TEV (Tobacco Etch Virus) protease gained particular popularity. The protein of interest and the fusion tag could be engineered to share the TEV recognition sequence (ENLYFQG/S), enabling targeted cleavage without compromising the target protein. Other frequently used cleavage enzymes included enterokinase, thrombin, and factor Xa; each had

unique recognition sequences and ideal circumstances. The target protein's amino acid sequence, the necessary reaction conditions, and the existence of internal cleavage sites all influenced the protease selection. The creation of His-tagged proteases made it simple to remove them following cleavage reactions, which streamlined the last stages of purification.

ADDRESSING PROTEIN FOLDING CHALLENGES

Cold-shock promoters and low-temperature expression systems were developed to address protein misfolding issues, particularly for eukaryotic proteins expressed in bacterial hosts(Qing et al., 2004).The recognition that protein folding kinetics often competed unfavorably with aggregation kinetics at standard expression temperatures led to strategies for slowing translation rates and reducing protein synthesis temperatures. The cold-shock response in bacteria involves the induction of specific genes that help cells adapt to lower temperatures, and some of these promoters showed activity at reduced temperatures where protein folding was more favorable. The development of chaperone co-expression systems represented another approach to improving protein folding. Expression vectors could be co-transformed with plasmids that encode molecular chaperones like GroEL/ES, DnaK/DnaJ/GrpE systems, or specialized folding catalysts like protein disulfide isomerase. Although they frequently resulted in lower total protein yields, these systems helped with appropriate protein folding and decreased the formation of inclusion bodies. By shielding the recombinant protein from proteolytic degradation and streamlining the initial purification processes, inclusion body formation may actually be beneficial for some proteins, especially those intended for refolding or chemical modification. This method is now feasible for industrial protein production thanks to the development of specialized protocols for inclusion body isolation and protein refolding.

OPTIMIZATION OF BACTERIAL EXPRESSION CONDITIONS

The process of optimizing bacterial expression conditions necessitates meticulous evaluation of several factors, such as temperature, the timing of induction, the concentration of inducers, and the composition of the culture medium (Baneyx & Mujacic, 2004). Temperature represents a particularly critical variable, as elevated temperatures can enhance protein expression rates while simultaneously increasing the risk of protein aggregation and inclusion body formation (Schein & Noteborn, 1988). A major influence on the yield and quality of recombinant protein products is the timing of induction in relation to the culture growth phase. Although it may jeopardize protein folding and solubility, early induction during the exponential growth phase usually maximizes total protein yield (Donovan et al., 1996). Improving protein solubility and biological activity through late induction, which is started during the late exponential or early stationary phase, frequently comes at the price of overall yield (Makrides, 1996).

MAMMALIAN EXPRESSION SYSTEMS

The shortcomings of prokaryotic and yeast systems were addressed by the development of mammalian expression systems, especially for proteins that need intricate post-translational modifications. Viral promoters like the cytomegalovirus (CMV) immediate early promoter and the SV40 early promoter were used in the first mammalian expression vectors, and they produced robust, constitutive expression in mammalian cells (Gorman et al., 1982).

The development of calcium phosphate transfection and electroporation methods enabled efficient delivery of expression plasmids into mammalian cells (Graham & van der Eb, 1973) (Neumann et al.,

1982). The incorporation of selectable markers such as neomycin resistance (neo^r) and hygromycin resistance (hyg^r) allowed for the isolation of stable cell lines expressing recombinant proteins.

INDUCIBLE MAMMALIAN SYSTEMS

The tetracycline-inducible expression system, introduced by Gossen and Bujard in 1992, marked a significant advancement in mammalian expression technology, offering precise and reversible regulation of gene expression. This system utilized a tetracycline-controlled transactivator (tTA) that binds to tetracycline response elements (Ondov et al.) in the absence of tetracycline, activating transcription. The integration of tetracycline or doxycycline quickly and temporarily suppresses gene expression, highlighting a significant advancement in genetic studies. Further enhancements were achieved with the creation of the reverse tetracycline transactivator (rtTA), a crucial tool that enables gene expression to be actively induced when doxycycline is present, offering researchers more practical and precise control over experimental conditions (Gossen et al., 1995). These systems enabled the study of essential genes and toxic proteins that could not be constitutively expressed.

ADVANCED MAMMALIAN EXPRESSION FEATURES

The development of internal ribosome entry sites (IRES) enabled the expression of multiple proteins from a single transcript, facilitating the co-expression of proteins and selection markers (Jang et al., 1988). Self-cleaving peptide sequences, such as 2A peptides, provided an alternative approach for polycistronic expression with more predictable stoichiometry (Ryan et al., 1991). Tissue-specific and cell-type-specific promoters were incorporated into mammalian expression vectors to achieve targeted expression patterns. For instance, the muscle-specific promoters such as muscle creatine kinase and myosin heavy chain, alongside neuron-specific promoters like synapsin and neurofilament, facilitated research focused on expression patterns specific to particular cell types (Rindt et al., 1993).

MODERN DEVELOPMENTS AND SYNTHETIC BIOLOGY

a) Synthetic promoters, designed with specific regulatory elements, offer precise control over gene expression in terms of levels, timing, and cellular specificity (Redden & Alper, 2015). These engineered systems often incorporate multiple regulatory inputs, enabling complex expression logic circuits.
b) The adaptation of CRISPR-Cas technology for transcriptional activation has created new paradigms for expression control. dCas9-based activator systems (CRISPRa) enable programmable gene activation through guide RNA targeting, providing flexible and multiplexable expression control (Gilbert et al., 2013). These systems offer advantages in terms of modularity and the ability to simultaneously regulate multiple genes.
c) Cell-free protein synthesis systems have increasingly become viable alternatives to traditional cell-based expression methods, which hold particular advantages in handling toxic proteins, enabling swift prototyping, and facilitating the integration of non-natural amino acids. Both prokaryotic and eukaryotic cell-free systems have been developed, with recent advances improving yield, reaction duration, and protein quality (Carlson et al., 2012).

PLASMID DEGRADATION AND RESTORATION TECHNIQUES

Plasmid degradation represents a significant challenge in both laboratory settings and clinical applications. The degradation can occur through multiple pathways, including enzymatic cleavage by nucleases, oxidative damage from reactive oxygen species, thermal denaturation, and physical

shearing during handling and storage (Molina, 2021). These degradation mechanisms can lead to linearization of circular plasmids, introduction of nicks and breaks, base modifications, and complete fragmentation of the DNA molecule. The expanding applications of plasmids heighten the importance of developing effective restoration techniques. While conventional methods have primarily aimed at preventing degradation by ensuring optimal storage conditions and applying protective additives, recent scientific progress has ushered in innovative enzymatic repair systems, chemical restoration techniques, and biotechnological strategies designed to actively mend damaged plasmid DNA (Sun et al., 2019).

MECHANISMS OF PLASMID DEGRADATION

NUCLEASE-MEDIATED DEGRADATION

Nucleases pose a significant threat to the integrity of plasmids, especially in samples contaminated with cellular debris or not properly purified (Green & Sambrook, 2012). These enzymes are fundamentally categorized as endonucleases, responsible for cleaving internal phosphodiester bonds, thereby inducing breaks that convert supercoiled plasmids into relaxed or linear configurations (Linn & Roberts, 1982), and Exonucleases operate by excising nucleotides from the terminal sections of DNA. The functioning of these nucleases is subject to a myriad of factors such as pH, temperature, ionic strength, and notably, the presence of divalent cations like Mg^{2+} and Ca^{2+} (Lazarus & Seaman, 2021). DNase I, in particular, necessitates Mg^{2+} or Mn^{2+} to achieve peak activity, and it operates effectively at physiological pH levels, highlighting its potential disruptive impact in biological systems.

NUCLEASE TYPE	MECHANISM	PRIMARY TARGET	EFFECT ON PLASMIDS	EXAMPLES
DNase I	Endonuclease	dsDNA	Random nicking/cleavage	Bovine pancreatic DNase I
DNase II	Endonuclease	dsDNA (acidic pH)	Double-strand breaks	Porcine spleen DNase II
Exonuclease III	3' to 5' exonuclease	dsDNA	Progressive degradation	E. coli ExoIII
BAL-31	Dual exo/endonuclease	ssDNA/dsDNA	Terminal degradation	Alteromonas espejiana
S1 Nuclease	Single-strand specific	ssDNA	Nick conversion	Aspergillus oryzae
Micrococcal Nuclease	Endonuclease	ssDNA/dsDNA	Non-specific Cleavage	Staphylococcus aureus

Table 1: Grouping of Nucleases Affecting Plasmid Stability

OXIDATIVE DEGRADATION

Reactive oxygen species (ROS) pose a significant threat to plasmid DNA through multiple oxidative pathways. Reactive oxygen species, notably affecting DNA, include hydroxyl radicals(•OH), superoxide anions ($O_2•^-$), hydrogen peroxide (H_2O_2), and singlet oxygen($^1O_2$) (Cadet et al., 2010). The oxidative damage manifests in several forms:

1. **Base Modifications**: The most common oxidative lesion is 8-oxo-7,8-dihydroguanine (8-oxoG), formed through guanine oxidation. This modification can lead to G:C to T:A transversion mutations during replication (Halliwell & Gutteridge, 2015). The accumulation of such lesions

over extended storage periods can lead to replication errors, strand breaks, and ultimately complete loss of plasmid functionality (Cadet et al., 2003).

2. **Sugar-Phosphate Backbone Damage**: Oxidation of the deoxyribose sugar can result in strand breaks, with the C1' and C4' positions being particularly susceptible to abstraction reactions.
3. **Cross-linking**: Bifunctional oxidants can create interstrand or intra-strand cross-links, severely compromising DNA structure and function.

The kinetic parameters for oxidative damage vary significantly based on the oxidant species and environmental conditions.

OXIDANT SPECIES	RATE CONSTANT ($M^{-1}S^{-1}$)	PRIMARY TARGET	MAJOR PRODUCTS
•OH	1×10^{10}	All bases/sugar	8-oxoG, strand breaks
$^1O_2$	1×10^{8}	Guanine	8-oxoG, spiroiminodihydantoin
$O_2^{\bullet -}$	1×10^{6}	Iron-DNA complexes	Site-specific breaks
H_2O_2	1×10^{3}	Metal-catalyzed	Fenton-type damage

Table 2: Kinetic Parameters for Oxidative DNA Damage

THERMAL DEGRADATION

Thermal stress affects plasmid stability through multiple mechanisms, including DNA melting, depurination, and acceleration of chemical degradation reactions. Plasmids exhibit thermal stability, which is defined by their melting temperature (Karp et al.), which is influenced by various factors, including the guanine-cytosine (G-C) content, ionic strength, and pH levels (Jin et al., 2018).

The Arrhenius equation describes the temperature dependence of degradation rates:

$$k = A \times e^{(-Ea/RT)}$$

Where k denotes the rate constant, A symbolizes the pre-exponential factor, Ea is the activation energy, R is the gas constant, and T is the absolute temperature. Depurination represents a particularly significant thermal degradation pathway, with an activation energy of approximately 30 kcal/mol for the glycosidic bond cleavage (Lindahl, 1974). This process generates apurinic (Qing et al.) sites that are highly unstable and prone to strand breakage.

MECHANICAL DEGRADATION

Plasmid degradation can occur due to physical forces such as shear stress, cavitation, and compression. The forces in question are frequently observed in activities such as pipetting, vortexing, sonication, as well as during freeze-thaw cycles.

The relationship between shear rate and DNA degradation follows the relationship:

$$\tau = \eta \times \dot{\gamma}$$

Where τ denotes the shear stress, η is the fluid viscosity, and $\dot{\gamma}$ is the shear rate.

High molecular weight DNA, including supercoiled plasmids, is particularly susceptible to mechanical degradation due to the increased hydrodynamic stress on longer molecules.

HYDROLYTIC DAMAGE

Under physiological conditions, continuous hydrolytic damage arises from the spontaneous breakdown of phosphodiester bonds and glycosidic linkages, a process exacerbated by increased temperatures, extreme levels of pH, and the presence of divalent metal ions (Friedberg et al., 2006).

Spontaneous Hydrolysis:

Reaction Mechanism: $R\text{-}PO_4^-\text{-}R' + H_2O \rightarrow R\text{-}PO_4H_2 + R'\text{-}OH$

Rate Factors:

- Temperature: It doubles with every 10°C rise in temperature
- pH: Maximum stability at pH 7.0-8.0
- Ionic strength: Metal ions catalyse hydrolysis
- Time: First-order kinetics for backbone cleavage

Quantitative Degradation Kinetics:

- **Half-life at 25°C, pH 7.0**: Approximately 30,000 years
- **Half-life at 95°C, pH 7.0**: Estimated to be approximately 30 minutes
- **Temperature coefficient**: For hydrolysis reactions, the coefficient, Q_{10} = 2-3
- **pH sensitivity**: 10-fold rate increase with each pH unit above 8.0

STORAGE CONDITIONS AND STABILITY FACTORS

The stability of plasmid DNA during storage is a matter of utmost importance, as it hinges on several critical environmental factors such as temperature, humidity, the composition of buffers, and the materials used for containers (Anchordoquy & Molina, 2007). For long-term preservation, storage at -80°C with suitable buffers generally ensures remarkable stability extending over years or even decades. Conversely, storage at 4°C poses a risk of gradual degradation over months to years, which largely depends on the conditions of the buffer . The choice of storage buffer significantly impacts plasmid stability, with Tris-EDTA (TE) buffer providing protection against both nuclease activity and metal-catalyzed oxidation through pH buffering and metal chelation respectively (Green & Sambrook, 2012). The addition of antioxidants like ascorbate or reduced glutathione is crucial in safeguarding against oxidative damage, especially concerning long-term storage needs . The selection of container materials and the conditions in which storage occurs play a pivotal role in affecting plasmid stability due to interactions with plastic surfaces, exposure to UV light, and potential contamination from atmospheric pollutants (Anchordoquy & Molina, 2007). Implementing low-binding plasticware and utilizing amber-colored containers are important measures to reduce surface interactions and prevent light-induced damage (Huber et al., 1993).

PLASMID RESTORATION TECHNIQUES

Plasmid restoration necessitates the deployment of various systems, determined by the extent and nature of the degradation observed. For minor degradation, it is often sufficient to carry out simple re-transformation into suitable bacterial hosts, followed by thorough colony screening and plasmid preparation. However, more significant degradation may require the use of molecular reconstruction techniques, such as PCR amplification of specific regions or even the complete re-synthesis of the plasmid (Green & Sambrook, 2012). Quality control measures for restored plasmids typically include restriction enzyme analysis, PCR verification of key regions, and complete sequencing of critical elements. Next-generation sequencing technologies have revolutionized plasmid verification by enabling rapid, comprehensive sequence confirmation at reduced costs (Goodwin et al., 2016). As repositories grow increasingly in both size and complexity, the establishment of standardized quality metrics for plasmid collections is essential. These metrics commonly encompass transformation efficiency, the accuracy of sequence fidelity, and thorough functional validation achieved through expression studies (Cozzi et al., 2017).

ENZYMATIC RESTORATION SYSTEMS

Enzymatic restoration represents the most sophisticated approach to plasmid repair, utilizing the cell's natural DNA repair machinery or engineered enzyme systems.

DNA LIGASE-BASED RESTORATION

T4 DNA Ligase: This enzyme exhibits broad substrate specificity and can ligate both cohesive and blunt-ended DNA fragments. The ideal reaction conditions involve maintaining a temperature of 16°C for a duration of 4 to 16 hours, while ensuring the presence of ATP and Mg^{2+} ions.

E. coli DNA Ligase: An enzyme reliant on NAD^+ predominantly enables precise joining of cohesive DNA termini.

The ligation efficiency can be calculated this way :

Efficiency = (Ligated molecules / Total molecules) × 100%

POLYMERASE-BASED RESTORATION

DNA polymerases have the capacity to rectify damaged plasmids by employing their 3' to 5' exonuclease activity, which serves as a proofreading mechanism allied with their polymerase activity. Key enzymes include:

T4 DNA Polymerase: Possesses strong 3' to 5' exonuclease activity, making it effective for removing damaged bases and filling in gaps.

Pfu DNA Polymerase: High-fidelity enzyme with proofreading capability, suitable for accurate restoration of coding sequences.

Klenow Fragment: Modified E. coli DNA Polymerase I lacking 5' to 3' exonuclease activity, useful for fill-in reactions.

MULTI-ENZYME RESTORATION SYSTEMS

Complete restoration often requires multiple enzymatic activities working in concert.

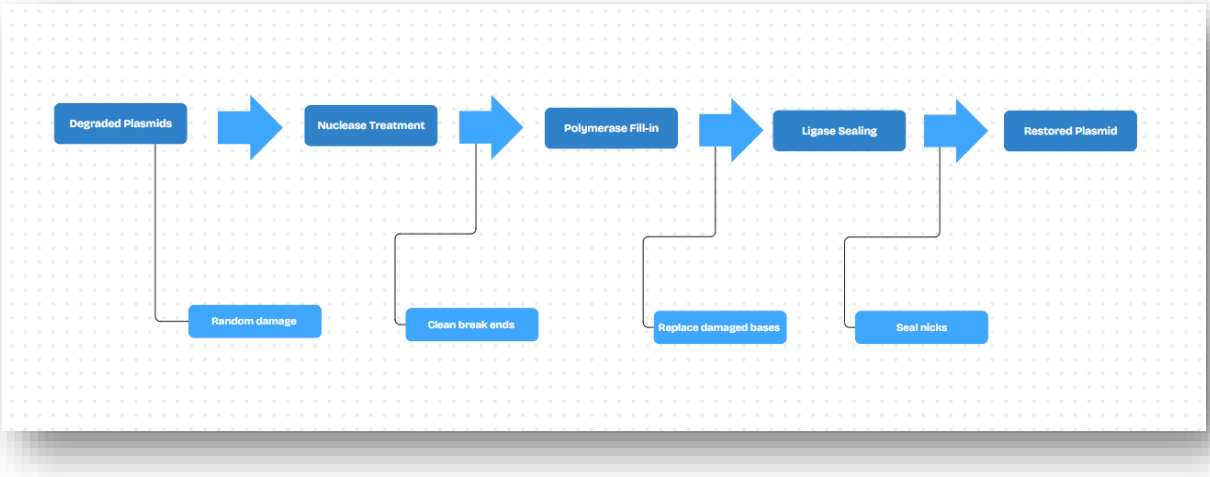


Figure 2: Schematic representation of multi-enzyme plasmid restoration pathway showing the sequential action of nucleases, polymerases, and ligases.

CHEMICAL RESTORATION METHODS

Chemical approaches to plasmid restoration focus on neutralizing damaging agents and providing chemical environments that favor DNA stability.

Antioxidant Systems:

Antioxidants prevent and reverse oxidative damage through various mechanisms:

Reducing Agents:

- Dithiothreitol (DTT): Reduces disulfide bonds and chelates metal ions
- β-Mercaptoethanol: Similar reducing properties with lower toxicity
- Tris(2-carboxyethyl)phosphine (TCEP): Non-thiol reducing agent

Radical Scavengers:

- Mannitol: Scavenges hydroxyl radicals with high efficiency
- Dimethyl sulfoxide (DMSO): Broad-spectrum radical scavenger
- Ascorbic acid: Water-soluble antioxidant effective against multiple ROS

Metal Chelators:

- EDTA: Chelates bind divalent cations, effectively inhibiting oxidation catalyzed by metals.
- Desferrioxamine: Specific iron chelator
- Bathocuproine: Copper-specific chelator

pH Buffering Systems:

- Maintaining optimal pH is crucial for plasmid stability, as extreme pH values can cause base deprotonation and strand separation. Common buffering systems include:

BUFFER SYSTEM	PKA	OPTIMAL PH RANGE	ADVANTAGES	LIMITATIONS
Tris-HCl	8.1	7.0-9.0	Good buffering capacity	Temperature sensitive
HEPES	7.5	6.8-8.2	Minimal temperature effect	Expensive
Phosphate	7.2	6.5-8.0	Physiologically relevant	Precipitates with divalent cations
Bis-Tris	6.5	5.8-7.2	Stable at low temperatures	Limited buffering range

Table 3: Buffering Systems for Plasmid Stabilization

PHYSICAL RESTORATION APPROACHES

Physical techniques aimed at plasmid restoration concentrate on fine-tuning environmental parameters and applying physical forces to ensure the correct configuration of DNA, underscoring the critical nature of these processes.

Thermal Annealing

Controlled thermal cycling can restore hydrogen bonding in partially denatured plasmids. The annealing process follows the relationship:

Rate of annealing $\propto \sqrt{C_0 t}$; (Where C_0 is the initial DNA concentration and t is time)

Optimal annealing conditions typically involve:

- Temperature: 65-68°C (5°C below Tm)
- Time: 30-60 minutes
- Salt concentration: 150-500 mM NaCl

Electrophoretic Restoration

Pulsed-field gel electrophoresis (PFGE) is a sophisticated method specifically crafted to effectively separate and recover intact plasmids from mixtures that contain fragmented or degraded DNA. The separation is based on size-dependent mobility differences under alternating electric fields.

Osmotic Stabilization

Osmotic agents help maintain DNA structure by controlling water activity and preventing excessive hydration or dehydration:

- ✓ **Polyethylene glycol (PEG)**: Molecular crowding agent that stabilizes DNA structure
- ✓ **Trehalose**: Disaccharide that forms hydrogen bonds with DNA
- ✓ **Glycerol**: Cryoprotectant that maintains DNA flexibility at low temperatures

COMPARATIVE ANALYSIS OF RESTORATION TECHNIQUES

EFFICACY COMPARISON

The effectiveness of different restoration techniques varies significantly based on the type and extent of damage.

Table 4: Assessing the Relative Effectiveness of Plasmid Restoration Methods

RESTORATION METHOD	NICK REPAIR	BREAK REPAIR	OXIDATIVE DAMAGE	BASE MODIFICATIONS	OVERALL SCORE
T4 DNA Ligase	95%	85%	20%	10%	52.5%
Multi-enzyme System	90%	90%	70%	60%	77.5%
Chemical Antioxidants	10%	5%	90%	30%	33.8%
CRISPR-based	80%	95%	40%	85%	75.0%
Thermal Annealing	60%	30%	15%	20%	31.3%

COST-BENIFIT ANALYSIS

Economic considerations are crucial for selecting appropriate restoration techniques.

Cost-Effectiveness Index = Success Rate / (Cost × Time)

METHOD	COST PER REACTION	SET UP	SUCCESS RATE	COST-EFFECTIVE INDEX
Enzymatic	$15-50	2-4 hours	70-90%	0.018-0.025
Chemical	$5-15	30 minutes	40-60%	0.013-0.019
Physical	$1-5	1-2 hours	30-50%	0.020-0.033
CRISPR-based	$100-300	8-12 hours	80-95%	0.003-0.009

Table 5: Cost-Benefit Analysis of Restoration Methods

INDEXING AND DATABASE MANAGEMENT SYSTEMS

SYSTEMATIC CATALOGUING APPROACHES

The systematic cataloguing of plasmid collections demands a standardized methodology that meticulously records crucial information yet remains sufficiently adaptable to encompass various vector types and applications (Karp et al., 2009). Implementing hierarchical classification systems, defined by vector backbone, insert type, and intended application, ensures an intuitive structure that significantly enhances researchers' abilities to search and browse efficiently (Miller & Calos, 2007). The development of standardized nomenclature systems enables consistent identification and communication regarding specific plasmid constructs across different laboratories and research institutions (Canton et al., 2008). The Registry of Standard Biological Parts represents one example of such standardization efforts, providing unique identifiers and standardized descriptions for genetic components used in synthetic biology applications. The design of a database schema must thoughtfully address the intricate relationships among plasmids, their constituent parts, and the associated experimental data, ensuring efficient query capabilities and maintaining data integrity constraints (Garcia-Hernandez et al., 2002). In essence, relational database models systematically arrange information into interconnected tables that represent plasmids, sequences, features, publications, and experimental results, reflecting the complexity and importance of these components (Karp et al., 2010).

MOLECULAR BARCODING AND RACKING SYSTEMS

Molecular barcoding systems provide a reliable method for plasmid identification, ensuring precise results irrespective of label condition and reducing human errors in record keeping (Brenner & Lerner, 1992). DNA barcoding techniques utilize distinct sequence identifiers integrated into plasmid constructs, allowing them to be swiftly sequenced or identified using PCR-based methods to verify plasmid identity (Hebert et al., 2003). The integration of barcoding systems with laboratory automation enables high-throughput processing and tracking of plasmid samples throughout preparation, storage, and distribution workflows. Robotic systems possess the capability to autonomously sequence barcode regions and update database records, significantly reducing the risk of sample mix-ups and ensuring precise inventory control. Two-dimensional barcode systems, like Data Matrix codes, are adept at encoding extensive data within compact formats, making them ideal for labeling small sample containers while remaining easily readable by conventional laboratory equipment (Kato & Tan, 2005). The implementation of these systems alongside laboratory information management systems (LIMS) facilitates thorough tracking of sample history and experimental usage, underscoring their critical role in laboratory operations (Thoma et al., 2001).

DIGITAL STORAGE AND RETRIEVAL SYSTEMS

Digital storage systems for plasmid information necessitate a thoughtful balance between accessibility, security, and long-term preservation, addressing the varied needs of research communities. While cloud-based platforms offer scalability, automatic backups, and multi-site accessibility, it is imperative to meticulously evaluate data security and ownership concerns (Armbrust et al., 2010). Moreover, the adoption of version control systems from software development practices serves as a crucial method for meticulously tracking changes in plasmid sequences and their associated documentation over time. It is imperative that search and retrieval algorithms are designed to accommodate the varied approaches researchers use to query plasmid databases, such as sequence-based searches, feature-

oriented questions, and literature-associated inquiries (Altschul et al., 1990). The implementation of full-text indexing and semantic search capabilities can significantly enhance the utility of plasmid databases for research applications (Manning et al., 2008)

QUALITY ASSURANCE AND STANDARDIZATION

ANALYTICAL METHODS FOR PLASMID CHARACTERIZATION

Plasmid in-depth characterization involves the implication of various methodological approaches and evaluation criteria focusing on structural integrity, purity, and function (Joseph Sambrook, 2012). Spectrophotometric analysis provides rapid assessment of DNA concentration and purity through measurement of absorbance at 260 nm and calculation of A260/A280 and A260/A230 ratios (Joseph Sambrook, 2012). Pure DNA preparations typically exhibit A260/A280 ratios between 1.8 and 2.0, with lower values indicating protein contamination and higher values suggesting RNA contamination or alkaline conditions. Fluorometric measurement methods, using DNA-specific fluorescent dyes (e.g., PicoGreen or SYBR Green) have a higher sensitivity and specificity than spectrophotometric methods and especially are useful for low-content samples . These procedures are less sensitive to contamination and more specifically quantitate in the presence of RNA or protein impurities. Capillary electrophoresis systems offer high-resolution analysis of plasmid preparations, enabling the detection and quantification of different topological forms including supercoiled, relaxed, and linear DNA. Automated capillary electrophoresis systems can process multiple samples rapidly while providing quantitative data on the relative proportions of different plasmid forms (Birren & Lai, 1994)

CONTAMINATION DETECTION AND PRESERVATION

Contamination in plasmid preparation and storage poses a serious and ongoing challenge, with possible sources being bacterial chromosomal DNA, RNA, proteins, endotoxins, and foreign plasmids (Joseph Sambrook, 2012). Bacterial chromosomal DNA contamination can be assessed through restriction enzyme analysis using enzymes that cut chromosomal DNA frequently but do not cut the plasmid of interest (Birnboim & Doly, 1979). RNA contamination can be detected through agarose gel electrophoresis and quantified using RNase treatment followed by spectrophotometric analysis. Plasmid preparation procedures that include RNase treatment effectively remove RNA contamination while retaining plasmid DNA (Birnboim & Doly, 1979). Endotoxin contamination is of particular importance for plasmids for use in mammalian cell culture or in mammalian in vivo applications, as extremely low levels can result in inflammatory responses and confound experiments. Limulus amebocyte lysate (LAL) assays provide sensitive detection of endotoxin contamination, with chromogenic and fluorogenic variants enabling quantitative analysis (Williams, 2019).

DATABASE ARCHITECHTURE AND DESIGN PRINCIPLES

This database has experienced several developmental stages in the progression of plasmid databases. Earlier systems like Plasmid Database at University of Wisconsin were more specialized, and were primarily focused on sequence storage, with manual annotation. With the genomics revolution that took place in early 2000s, more elaborate systems have been designed. The ACLAME database A Classification of Mobile genetic Elements introduced some of the first systematic classification of mobile genetic elements, such as plasmids, and set important standards in annotation (Leplae et al.,

2010). This era also witnessed the introduction of webforms, and the use of standardised data types like GenBank. Current plasmid databases have adopted high-throughput data processing and automated analysis pipelines. Systems, such as PLSDB and PlasmidFinder, are included in the current state of art and have implemented high-level sequence analysis algorithms and extensive web-based analysis tools (Carattoli et al., 2014; Galata et al., 2018)

The high sequence diversity of plasmids requires flexible data models capable of representing different types of genetic systems. There is a very wide range of plasmid size, unlike for chromoso- mes; from small cryptic plasmids (a few kilobases) to megaplasmids, in excess of 1 Mb (Garcillán-Barcia et al., 2009). In addition to the above, the modular architecture of plasmids creates additional challenges in the databases design. Plasmids often harbour mobile genetic elements, such as insertion sequences and transposons, which can dramatically change the content of the sequences via recombination. This dynamic nature requires database systems capable of tracking sequence relationships and evolutionary histories across related plasmid families. Data quality control represents another significant challenge. The increasing reliance on automated sequencing and assembly pipelines has introduced new sources of error, including chimeric sequences, assembly artifacts, and contamination from chromosomal DNA. Effective quality control mechanisms must balance the need for comprehensive data inclusion with the requirement for high-quality, reliable sequence information.

RELATIONAL DATABASE MANAGEMENT SYSTEMS

Relational database management systems (RDBMS) form the backbone of most contemporary plasmid databases due to their robust data integrity mechanisms and standardized query capabilities. PostgreSQL and MySQL are the most commonly employed platforms, chosen for their scalability, ACID compliance, and extensive optimization features. The relational model provides several advantages for plasmid data management. Normalized data structures eliminate redundancy and ensure consistency across related data elements. Modern relational database management systems offer advanced indexing features that significantly enhance the efficiency of query processing across expansive datasets. B-tree indexes afford logarithmic time access to sequence records, which is vital for swift retrieval. Meanwhile, specialized index types, such as Generalized Search Tree (GiST) indexes, are essential in managing complex geometric and text-based queries frequently utilized in bioinformatics, underscoring the depth and precision required in handling and analyzing intricate data structures. Relational systems exhibit certain limitations when managing plasmid data, as their stringent schema requirements can be restrictive when handling diverse annotation formats or variable experimental metadata. Furthermore, they may encounter performance issues with complex queries requiring multiple joins across extensive tables, especially during sequence similarity operations.

NoSQL DATABASE APPROACHES

Document-oriented databases like MongoDB are increasingly adopted for managing plasmid data, especially where flexible schema design and horizontal scaling are needed. The document model effectively supports the hierarchical structure of genomic annotations and facilitates the efficient storage of diverse metadata formats (Garcillán-Barcia et al., 2009). Notable advantages of NoSQL approaches include schema flexibility, allowing for dynamic adaptation to evolving data formats and annotation standards. Native JSON functionality enhances data exchange with web-based applications and bioinformatics platforms. The ability to scale horizontally allows for distribution across multiple servers, improving execution speed for extensive analytical tasks. Graph databases, such as Neo4j and Amazon Neptune, are increasingly adopted for managing plasmid data due to their ability to effectively depict intricate evolutionary ties and networks of sequence similarity. These databases offer

specialized graph query languages refined for navigating the complex relational constructs typical in comparative genomics studies.

HYBRID ARCHITECTURES

Several modern plasmid databases use hybrid architectures that integrate various database technologies to enhance performance for diverse data types and query patterns. Such designs commonly incorporate a relational database for structured metadata and sequence identifiers, alongside NoSQL systems for flexible annotation data, and graph databases for mapping relationships. These hybrid approaches allow for the optimization of distinct components tailored to specific use cases, while ensuring system coherence through clearly defined APIs and data synchronization protocols. Nevertheless, they also add layers of complexity concerning system administration, data consistency management, and query optimization across various platforms.

MAJOR PLASMID DATABASE SYSTEMS

PLSDB (PLASMID DATABASE)

PLSDB serves as one of the most extensive modern plasmid databases, encompassing more than 15,000 complete plasmid sequences with standardized metadata and automated annotation processes (Galata et al., 2018). It operates on a PostgreSQL backend, enhanced with custom indexing optimizations tailored for genomic data. The database's architecture integrates numerous advanced features, including automated quality control workflows, standardized systems for taxonomic classification, and detailed profiling of resistance genes. The annotation pipeline incorporates a suite of advanced prediction tools, such as Prokka for gene prediction, BLAST for conducting homology searches, and various specialized tools for the detection of mobile elements. PLSDB's indexing strategy combines traditional B-tree indexes for metadata queries with custom sequence indexes optimized for similarity searching. The web interface offers advanced search capabilities, including sequence similarity searches, metadata filtering, and batch download options. Additionally, RESTful APIs facilitate programmatic access for high-throughput applications and integration with third-party analytical tools.

PlasmidFinder DATABASE

PlasmidFinder is a specialized tool dedicated to the identification and typing of plasmids via a meticulously curated database of plasmid replicon sequences (Carattoli et al., 2014). The system is designed with an efficient architecture that enhances rapid sequence classification and facilitates real-time analysis through web-based applications. At its core, the database comprises thoughtfully curated replicon sequences that represent key plasmid incompatibility groups. Each sequence is linked to detailed metadata, encompassing incompatibility group classifications, host range specifics, and relevant literature references. Domain experts manually conduct the curation process to ensure the reference sequences are of the highest quality. The analytical pipeline utilizes BLAST-based similarity searches with meticulously optimized parameters for effective plasmid classification. Integration with whole-genome sequencing workflows facilitates the automatic detection of plasmids within bacterial genome assemblies. The web interface of PlasmidFinder is carefully designed to be user-friendly, providing efficient input methods and presenting results in a clear and concise manner. The system accommodates both individual sequence queries and batch processing to support high-throughput applications.

COMPASS (COMPARATIVE PLASMID SUITE)

COMPASS (Comparative Plasmid Suite) COMPASS offers an extensive platform for plasmid comparison and analysis, integrating various database backends and specialized analytical tools . Designed with a microservices architecture, the system allows for modular deployment and tailored optimization across different analytical capacities. The database component is built on a hybrid architecture that merges relational and NoSQL technologies, with primary sequence and metadata housed in PostgreSQL databases, while dynamic annotation data and analytical outcomes are stored in MongoDB collections. This method allows for optimization tailored to various query patterns while preserving the system's overall coherence. It offers support for multiple sequence alignment algorithms and includes integrated visualization tools for conducting comparative analyses.

ESTABLISHED INDEXING STRATEGIES AND ALGORITHMS

SEQUENCE-BASED INDEXING

1. k-mer INDEXING APPROACHES

k-mer indexing has emerged as a fundamental technique for rapid sequence similarity searching in plasmid databases. This approach decomposes sequences into overlapping substrings of fixed length k, enabling efficient approximate matching and classification (Lock et al., 2018). The selection of the k-value is a crucial optimization parameter, wherein lower values enhance sensitivity but compromise specificity. Advanced k-mer indexing systems such as Kraken2 employ taxonomic classification trees to enable rapid species-level identification of query sequences. The system efficiently creates a concise hash table that associates k-mers with taxonomic identifiers, allowing the classification of millions of sequences per hour using standard hardware (Lock et al., 2018). MinHash algorithms offer a different method for k-mer indexing by generating compact sketches that maintain sequence similarity relationships and significantly decrease storage needs. Tools like Sourmash employ MinHash sketching to conduct comprehensive sequence comparisons, enabling effective clustering and similarity searches across large collections of sequences (Brown & Irber, 2016).

2. SUFFIX ARRAYS AND SUFFIX TREES

Suffix arrays and suffix trees provide exact pattern matching capabilities essential for identifying conserved sequences and regulatory elements within plasmid collections. These data structures enable efficient identification of a given pattern within a sequence database, with query times proportional to the pattern length rather than the database size (Gusfield, 1997). Contemporary methods for constructing suffix arrays utilize space-efficient algorithms and compressed formats to reduce memory usage. The Burrows-Wheeler Transform offers a notably effective technique for developing compressed suffix arrays, making it well-suited for extensive genomic databases.

FEATURE-BASED INDEXING

1. GENE ANNOTATION INDEXING

The comprehensive indexing of functional annotations facilitates complex queries that integrate sequence similarity with functional attributes. Contemporary plasmid databases possess specialized

indexes for various annotation categories, such as antibiotic resistance genes, virulence factors, mobile genetic elements, and metabolic pathways. The ResFinder database serves as a prime example of advanced annotation indexing, as it maintains curated collections of antibiotic resistance genes along with metadata covering resistance mechanisms, antibiotic specificities, and clinical relevance (Zankari et al., 2012). The indexing system facilitates rapid detection of resistance determinants within query sequences and supplies thorough metadata for analysis. Analogous indexing methodologies are employed by virulence factor databases, such as VFDB, for pathogenicity-associated genes, allowing for a structured investigation of virulence potential across plasmid collections. These advanced indexes typically adopt hierarchical classification schemes, capturing the intricate interconnections between gene families and their functional categories.

2. STRUCTURAL FEATURE INDEXING

Plasmid structural features including origin of replication sequences, partition systems, and conjugation apparatus represent important indexing targets for specialized analyses. Despite overall divergence in sequence, these features exhibit conservation, requiring the implementation of specialized indexing strategies. Hidden Markov Models (HMMs) deliver efficient instruments for indexing structure features defined by sequence motifs with variable conservation patterns. Databases like Pfam offer pre-curated HMM libraries for protein families typically linked with plasmid functions (Finn et al., 2016).

3. METADATA INDEXING

Contemporary systems use multi-dimensional indexing models that support various types of metadata such as taxonomic classifications, sources of isolation, geographic origins, and experimental conditions. These strategies often utilize ontology-based indexing approaches that apply standardized vocabularies, thereby facilitating semantic queries across diverse metadata collections. The Gene Ontology serves as a hierarchical classification system delineating biological processes, molecular functions, and cellular components (Gene Ontology Consortium, 2019). The integration of ontological indexing allows for advanced queries, including the identification of plasmids that contain genes participating in particular metabolic pathways or stress responses.

DATA INTEGRATION AND STANDARDIZATION

ONTOLOGY INTEGRATION

The Gene Ontology presents a structured approach to functional annotation, enabling the systematic classification of functions encoded by plasmids, as outlined by the Gene Ontology Consortium in 2019 (Ferrer-Miralles et al.). Concurrently, the Antibiotic Resistance Ontology offers a specialized lexicon for understanding resistance mechanisms, thereby allowing precise classification of resistance determinants and associated phenotypes (Jia et al., 2016). This ontological structure supports complex queries that integrate genetic and phenotypic data, thus enabling exhaustive profiling of resistance. The Sequence Ontology (SO) establishes standardized terminology for genomic features to ensure consistent annotation frameworks across various database systems (Eilbeck & Lewis, 2004). By integrating SO terms, automated annotation pipelines gain interoperability, enhancing data integration across numerous platforms.

DATA FORMAT STANDARDIZATION

Standardized data formats are essential for achieving interoperability among various plasmid database systems. The GenBank format continues to be the leading standard for exchanging sequence data, offering a well-organized depiction of sequences along with their associated annotations (Benson et al., 2018). Meanwhile, the Generic Feature Format version 3 (GFF3) provides a more adaptable framework for representing genomic annotations, fostering hierarchical feature relationships and supporting systems with extensible attributes (Sequence Ontology Project, 2016). To enhance interoperability, many modern systems are compatible with both GenBank and GFF3 formats. JSON-LD, short for JavaScript Object Notation for Linked Data, is increasingly recognized as a standard format for representing interconnected biological data. Its adoption enhances the integration of semantic web technologies and promotes automated data discovery. This format supports assimilation into larger scientific data ecosystems, permitting automated discovery of relationships across various data types.

API DEVELOPMENT AND INTEROPERABILITY

RESTful APIs are crucial elements in contemporary plasmid databases, facilitating programmatic access and integration with analytical pipelines. They usually offer endpoints for retrieving sequences, querying metadata, and accessing analytical services, adhering to standard HTTP protocols to ensure a uniform interface design. The Bioschemas initiative promotes standardized schemas for representing biological data on the web, thus enhancing automated data discovery and integration. The Bioschemas markup implemented in plasmid databases facilitates search engine indexing and the automated harvesting of the data for meta-analyses. GraphQL is an emerging alternative to REST APIs, offering more flexible querying possibilities and allowing clients to only retrieve the data needed for their analyses. Several bioinformatics platforms have started adopting GraphQL for its superior performance character and developer's experience.

MACHINE LEARNING INTEGRATION

AUTOMATED CLASSIFICATION SYSTEMS

Machine learning algorithms have transformed plasmid classification and annotation by facilitating the automated analysis of large-scale sequence data. Support Vector Machines (SVMs) have been especially successful at predicting plasmid hosts, with accuracy rates over 90% on various training datasets (Arredondo-Alonso et al., 2017). Random Forest algorithms provide excellent classification capabilities for complex multi-class challenges, such as predicting plasmid mobility. These ensemble techniques blend several decision trees to produce high-accuracy predictions with interpretable feature importance rankings that uncover the sequence features most informative of certain phenotypes (Jin et al., 2018). Neural network methodology, especially deep learning topologies, also have been successful in automated plasmid annotation and functional prediction. Convolutional Neural Networks (CNNs) are capable of recognizing sequence motifs and structural motifs that are significant for plasmid function, whereas Recurrent Neural Networks (RNNs) are particularly good at handling variable-length sequence data (Asgari & Mofrad, 2015).

SIMILARITY CLUSTERING

Advanced clustering algorithms enable systematic organization of plasmid collections based on sequence similarity, functional content, and evolutionary relationships. Hierarchical clustering

techniques offer tree-based intuitive models of sequence relationships, while k-means clustering offers computationally optimal techniques for partitioning large-scale data. UMAP and t-SNE offer computationally efficient dimensionality reduction techniques for visualization of high-dimensional sequence data in two or three dimensions (McInnes et al., 2018). These techniques offer the capability to detect sequence clusters and outliers that could be indicative of new plasmid families or sequencing errors. Graph-based clustering techniques like Leiden clustering offer high performance for network data, allowing identification of highly connected sequence clusters without sacrificing computational efficiency for large-scale data.

Machine learning algorithms allow prediction of plasmid properties from sequence data only, reducing dependence on experimental validation for first-order predictions of function. Host range prediction algorithms combine sequence properties with known associations to predict prospective bacterial hosts for new plasmid sequences (Arredondo-Alonso et al., 2017). Prediction of conjugation potential is another important application, with algorithms trained on representative conjugative and non-conjugative plasmids precisely predicting transfer ability from sequence content (Smillie et al., 2010). Prediction is particularly valuable for prediction of horizontal gene transfer capacity in environmental communities. Stability prediction algorithms predict plasmid maintenance traits from presence and arrangement of replication and partition systems. These algorithms provide insight into plasmid ecology and evolution processes, guiding plasmid persistence research in bacterial populations.

QUALITY CONTROL AND VALIDATION

DATA VALIDATION FRAMEWORKS

Plasmid databases can only be efficient when comprehensive multidisciplinary quality control systems have been implemented. An automated validation pipeline checks the quality of the sequences with respect to assembly completeness, level of contamination, and consistency of annotations. There is a remarkable quality that is provided within genomes and plasmid assemblies which is based on single-copy gene presence and contamination analysis through CheckM (Parks et al., 2015). The system also detects possible chimeric sequences and assembly errors, which can be critical for many downstream analyses. Prokka also provides a standard annotation pipeline PGAP (Prokaryotic Genome Annotation Pipeline) that also incorporates automated quality control operational procedures. Such systems check the coherence of the prediction of specific genes, functional annotation of the genes, and cross-referencing in the databases and so flag possible errors in the annotations.

CONTAMINATION DETECTION, VERSION CONTROL AND PROVENANCE

Contamination poses a severe problem in plasmid sequence databases, having possible origins in the co-purification of chromosomal DNA, sample-to-sample contamination, as well as chimeric assemblies. Profiling sequences with metagenomics tools as Kraken2 and MetaPhlAn can determine if the sequence contains DNA fragments of several bacterial species, which hints at contamination (Beghini et al., 2021). Those provide resolution on species level which makes it possible to pinpoint the source of contamination. Another approach to detecting contamination is provided by similarity of sequences through graphs, that cross-check unique detected sequences against a wider database to trace patterns of unintended similarities which suggests contaminated data or cross contamination

(Ondov et al., 2016). Such methods are especially good in the case of systematic contamination of many records of sequences.

Current plasmid databases apply sophisticated version control systems that track changes to data. Changes made to schemas, updated data, and other steps in the analytical pipeline are meticulously recorded on the Git-based versioning system. All steps of data processing are tracked by a sysytem and thus repetition of the process is made possible, allowing errors which have been identified to be tracked and malfunctions to be resolved. A vocabularly PROV-O enabled within the framework of a device for automated information processes outlines the domain which in turn enables representation of a data's reasoned history in a computational way. The use of blockchai, provides new opportunities for unfalsified history tracking, safeguarded by cryptographic codes that verify sets of data and analytical workflows.

VISUALIZATION AND USER INTERFACES

INTERACTIVE WEB INTERFACES

Modern plasmid databases provide sophisticated web interfaces to address diverse user needs, ranging from browsing to sophisticated analysis. Dynamic layouts are tablet, desktop, and mobile enabled. Dynamic searching includes live suggestions and filtering capabilities, enabling users to rapidly limit the number of hits. Autocomplete speeds searching from controlled vocabularies and previous searches. Phylogenetic tree viewers support zooming for examining evolutionary relationships. Network visualization tools facilitate sequence similarity and functional relationship analysis.

GENOME BROWSERS AND SEQUENCE VISUALIZATION

With integrated genome browsers, research scientists can access individual plasmid sequences and their comprehensive annotation tracks. In contemporary browsers, multiple layers of annotations, including but not limited to genes and their predictions, functional domains, regulatory constituents, and experimental data tracks, are supported. JBrowse and IGV exemplify more flexible custom genome browsers with more general use acceptance (Buels et al., 2016). These browsers enable data streaming and support plugin frameworks which allows for integration of additional specialized logical instruments. With circular topologies being the preferred structural form for plasmids, circular genome viewers are of utmost importance for plasmid visualization. Circos and other similar tools are able to create complex circular diagrams that are multilayered and allow for data comparison.

STATISTICAL DASHBOARDS AND ANALYSIS

Statistical dashboards commonly present data regarding taxonomic composition, the predominance of functional categories, and the distribution of sequence lengths. Real-time data updating capabilities allow for the production of complex statistical visualizations using interactive charting tools like D3.js and Plotly (Bostock et al., 2011); **Plotly Technologies Inc., 2015**. Data production rates and research trends are revealed via time series visualizations, which monitor database growth and submission trends. Patterns of research activity and sample bias are revealed by using geographic visualizations to visualize the global distribution of sequence submissions.

CLOUD COMPUTING AND SCALABILITY

Cloud computing platforms have successfully transformed plasmid database scalability and access through the availability of elastic computing resources and global distribution capabilities. Amazon Web Services (AWS), Google Cloud Platform (GCP), and Microsoft Azure offer end-to-end database services optimized for different patterns of workload. Amazon RDS and Google Cloud SQL provide managed relational database services with automatic backup, scaling, and maintenance capabilities. These services support quick deployment of database systems with high availability without the need for specialized database administration expertise. NoSQL cloud services such as Amazon DynamoDB and Google Firestore provide high scalability for document-oriented workloads, supporting millions of operations per second with elastic automatic response to demand patterns.

Docker containerization, which offers uniform settings for development, testing, and production deployments, has emerged as the industry standard for plasmid database system deployment(Merkel, 2014). Containers remove environment-specific deployment problems by encapsulating application dependencies and configuration. The advanced container management features offered by Kubernetes orchestration platforms include fault tolerance, load balancing, and autonomous scaling. The implementation of intricate multi-component database systems with little operating overhead is made possible by these platforms. In order to improve overall system dependability through fault isolation, microservices architectures break down database systems into separate services. This allows for the independent scalability and optimization of various system components.

High performance at scale is achieved by cloud-native database designs. Static content can be distributed globally through Content Delivery Networks (CDNs), which lower latency for users in other countries and increase system responsiveness in general. Multiple-level caching techniques decrease database load and enhance query performance. Application-level caching minimizes redundant calculations, whereas Redis and Memcached offer high-performance in-memory caching for frequently accessed data. Database sharding divides data among several servers according to certain standards, including taxonomic classifications or sequence properties. This method preserves query performance while allowing horizontal scaling beyond the constraints of individual server hardware.

MATERIALS AND METHODS

This methodology outlines comprehensive and systematic approaches for the recovery and restoration of bacterial and mammalian plasmids that have been compromised by various forms of degradation, including nicks, breaks, base modifications, and structural alterations. The protocols detailed here are crucial for the assessment, purification, repair, and validation processes necessary to ensure successful plasmid restoration.

MATERIALS AND EQUIPMENTS

Table 6: DNA ISOLATION AND ANALYSIS REAGENTS

MATERIALS/ REAGENTS	COMPOSITION/ SPECIFICATION	SUPPLIER/ BRAND
DNA ISOLATION (MINI PREP/MIDI PREP) KIT	Proprietary formulation	QIAGEN, GENES2ME, SPINeasy
ISOPROPANOL	Analytical Grade	Sigma-Aldrich
ETHANOL	70% & Absolute	Sigma-Aldrich
RNase A	20 mg/mL	HiMedia
Agarose	Molecular Biology Grade	Figure 3: HIMEDIA (MB002-50G);Agarose

DNA Loading Dye	**10X concentration**	**Figure 4: TaKaRa; Loading Dye**
DNA Ladder	**1kb Ladder**	**Figure 5: Abclonal (RM30291); DNA Ladder**
Ethidium Bromide	**10 mg/mL stock**	**Sigma-Aldrich, HiMedia**

LYOPHILIZATION AND BLOTTING MATERIALS

- MCTs (1.5 ml)
- Aluminium Foil
- Mini Trans-Blot Accessories (**BIO-RAD)**
- Storage Bags (Ziploc Bags)
- Parafilm

Figure 6: BIO-RAD ; Trans-Blot Transfer Membranes

Table 7 : EQQUIPMENT AND INSTRUMENTATION

BASIC BALORATORY EQUIPMENT	
EQUIPMENT	**MODEL / SPECIFICATIONS**
Microcentrifuge	**Eppendorf (Centrifuge 5418 R)**

Figure 7: Eppendorf 5418 R

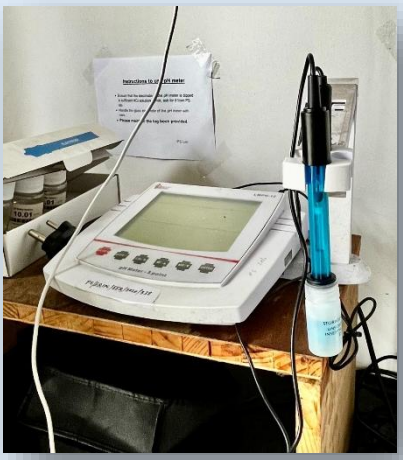
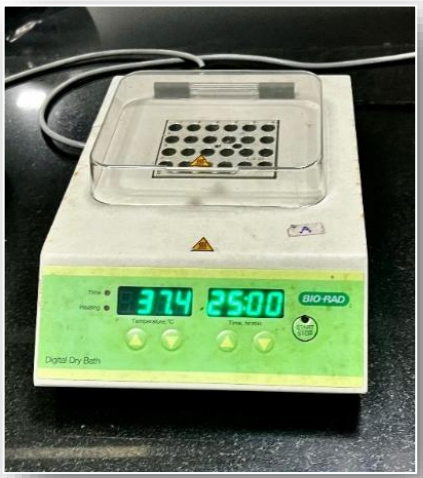
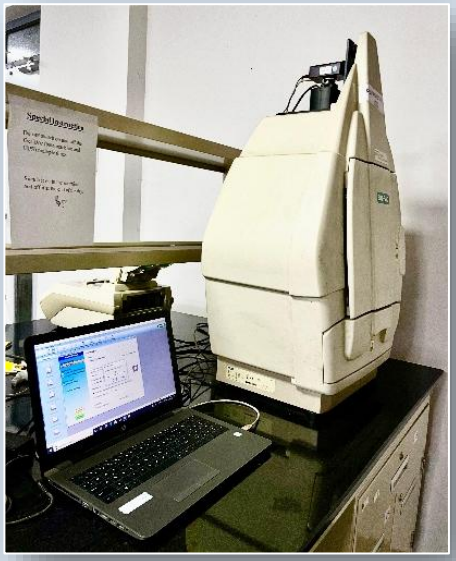
Centrifuge	**Eppendorf (Centrifuge 5910 R)** **Figure 8: Eppendorf 5910 R**
Vortex Mixer	**REMI (CM 101)** **Figure 9: REMI Cyclo Mixer 101**
Micropipettes	**Thermo Scientific (P10, P20, P200, P1000)**
Pipette Tips	**Corning**
pH Meter	**LMPH-12**  **Figure 10: LMPH1 pH Meter**
Analytical Balance	**Sartorius**

	Figure 11: Sartorius Practum 224-1S (220g-0.1mg) Analytical Balance
Water Bath	**Thermo Scientific** **Figure 12: Thermo Water Bath**
Heating Block	**BIO RAD** **Figure 13 : BIO-RAD Digital Dry Bath** 

SPECIALIZED EQUIPMENTS	
EQUIPMENT	**MODEL / SPECIFICATIONS**
Freeze Dryer	**EYELA (FDU-2110)** **Figure 14 : EYELA FDU-1110 Freeze Dryer**
Spectrophotometer	**Eppendorf** **Figure 15: Eppendorf BioPhotometer D30**

Gel Electrophoresis System	**Bio-Rad** **Figure 16: BIO-RAD Mini Protean Tetra System**
UV Transilluminator	**Bio-Rad**  **Figure 17: BIO-RAD ChemiDoc MP**
Autoclave	**TOMY SX-700**
Laminar Flow Hood	**LABWIT (ZHJH-C1109C)**
Incubator	**PRIME**

STORAGE EQUIPMENT	
EQUIPMENT	**MODEL / SPECIFICATIONS**

Refrigerator (4°C ± 2°C)	**TRUSFROST** **Figure 18:TruFrost Upright Medical Freezer,310 Ltrs**
Freezer (-20°C ± 5°C)	**ELANPRO**
Ultra-low Freezer (-80°C ± 10°C)	**U410 HEF**

Table 8: CONSUMABLES AND SUPPLIES

ITEMS	SPECIFICATION
Microcentrifuge Tubes	**1.5, 2.0 Ml**
Falcon Tubes	**15 mL, 50 Ml**
Culture Flasks (Conical)	**250 mL, 500 Ml**
Disposable Gloves	**Nitrile, powder-free**
Lab Coats	**Cotton/polyester blend**
Safety Goggles	**Chemical resistant**
Kimwipes	**Lint-free tissues**
Aluminum Foil	**Laboratory grade**
Permanent Markers	**Solvent resistant (Hydrophobic)**
Storage Boxes	**Plastic, compartmented (96 wells)**

EXPERIMENTAL DESIGN AND INDEXING FRAMEWORK

SAMPLE SELECTION AND RANDOMIZATION

Stock Selection Criteria:

- Age range: 2-15 years (stored at -80°C)
- Origin: Own laboratory source
- Vector types: Both bacterial and mammalian expression systems
- Storage conditions: Glycerol stocks in cryovials
- Replication: Each stock was processed in triplicate for statistical validation

GLYCEROL STOCK ASSESSMENT AND REVIVAL

Initial Assessment Protocol

Visual Inspection

I. Examined stored plasmid samples for:
   a. Colour changes (yellowing, browning)
   b. Precipitation or cloudiness
   c. Container integrity
   d. Storage condition documentation like Record stock age, storage temperature etc
   e. Photograph each stock for documentation

II. Removed stocks from -80°C storage

III. Allowed the stocks to thaw for precisely 15 minutes, adhering strictly to standardized timing guidelines.

IV. Use a calibrated thermometer to accurately document the temperature at which thawing occurs.

V. For each stock we inoculated 5-10ml LB broth with appropriate antibiotic. We added 50 µL glycerol stock culture using sterile pipette tip heads maintaining required environment.

VI. Maintained incubation at 37°C at a rotation speed of 225 rpm for 12-16 hours.

VII. Monitored OD_{600} at 2-hour intervals (Target OD_{600}: 0.6-1.2 for optimal plasmid yield) over 16 hours.

STANDARDIZED SPINeasy MINIPREP KIT PROTOCOL (FOR pDNA ISOLATION)

i. Harvest the overnight bacterial culture (OD_{600}= 0.8-1.2) by centrifuging at 5000g for 5 mins or 10000g for 3 mins. Supernatant was removed completely (verified by weight; recorded the pellet wet weight for normalization)

ii. Resuspended the bacterial pellet in 250µLof Resuspension Buffer SN1 (RNase A added). (Cells those were in a culture tube, transferred to a microcentrifuge tube)

Figure 19: SPINeasy DNA Plasmid Miniprep Kit

iii. Added 250 Alkaline Lysis Buffer SN2, mixed gently by inverting 4-6 times (No vortexing or rigorous pipetting should be employed) and incubated at room temperature (15-25°C) for 2 mins (not more than 5 mins).
iv. Added 350 µL of prechilled Neutralization Buffer P3, mixed well by multiple invertion for 4-6times, followed by an incubation on ice for 5 min (vortexing or pipetting is restricted).
v. Centrifuged at 14000-18000g for 10 min at 4°C.
vi. Transferred all of the lysate supernatant to a Column SN with collection tube.
vii. Centrifuged at 14000g for 1 min, discarded the flow through and reused the collection tube.
viii. Added 500 µL of Wash Buffer NW1 to the column.
ix. Centrifuged at 14,000g for 1 min and discarded the flow through and again reused the collection tube.
x. Added 750 µL of Wash Buffer NW2 to the column, followed by an incubation for 1 min at room temperature.
xi. Centrifuged at 14,000g for 1 min and discarded the flow through and reutilized the collection tube.
xii. Another round of Centrifugation at 14,000g for additional 1 min is employed to dry the column completely.
xiii. Removed the collection tube and placed column into a clean 1.5 ml MCT.
xiv. Added 50 µL of Elution Buffer SN to the center of the membrane and incubated at room temperature for 10 mins.
xv. Centrifugation for one round was done at 8000g for 1-2 mins to elute the DNA.
xvi. Eluted pDNA will be collected in the associated microcentrifuge tube.
xvii. Noted any unusual colour or turbidity. Eluted pDNA was stored immediately at 4°C for analysis.

DNA QUANTIFICATION AND PURITY ANALYSIS

I. Calibrated the Spectrophotometer with nuclease-free water or Elution Buffer whichever is used in mean times.
II. Measured 2 µL of each sample one by one.
III. Recorded A_{260}/A_{280} ratio, A_{260}/A_{230} ratio and absorbance.

AGAROSE GEL ELECTROPHORESIS QUALITY ASSESSMENT

I. Gel Preparation (0.8-1% Agarose)

a. Dissolved 0.4 gm / 0.5 gm in 50 mL 1X TBE/TAE Buffer
b. Heated until completely dissolved through microwave (2 minute intervals)
c. Applied 10 µL of EtBr dye, ensuring appropriate safety measures by wearing gloves during the application process.
d. Poured the whole liquid into the gel tray with 1.5 mm comb (comb size varied based on loading volumes)
e. We allowed it 30-40 minutes to polymerize at room temperature.

II. Sample Preparation and Loading

a. Mixed 3-4 µL DNA with 10X Loading Dye (1:6 ratio)
b. Loaded 10-12 µL sample-dye mixture per well.
c. Loaded samples in randomized order to prevent bias.

III. Started the run at constant 100V for approximately 45 minutes.

IV. **We used 1X TBE Buffer (pH 8.0) as Running Buffer. (Subsequently transferred to the TAE Buffer for temporary storage due to time constraints)**
V. **The complete migration was keenly monitored, utilizing the forefront of leading loading dye with proficiency and precision.**
VI. **Paused/Stopped when Dye reached 80-85% of Gel Length.**
VII. **Image Analysis And Scoring:**
   a. Captured image using ChemiDoc System.
   b. Used identical exposure settings (0.5 seconds).

LYOPHILIZATION PROCESS AND CYCLE DEVELOPMENT

I. Prepared lyoprotectant solutions in nuclease-free water.
II. Filter sterilization was done using 0.22 µm sterile syringe filter.
III. Stored at 4°C for short-term use
IV. Combined the purified plasmid DNA with the Lyoprotectant solution in equal volumes, maintaining a 1:1 ratio.
V. Gently pipetted to prevent DNA shearing.
VI. Aliquoted the plasmid DNA-lyoprotectant mixture into sterile, nuclease free microcentrifuge tubes which we sealed with paraffin slices with some sort of holes in it to maintain ventilation (volume varied as we eluted the pDNA in various amounts).
VII. To standardize the drying conditions we'll separate similar volumes in a different row later.
VIII. Placed samples in a -80°C freezer overnight to freeze completely.
IX. Transferred those frozen samples to the lyophilizer (maintained vertical orientation during freezing) ; Set the vacuum to ~0.1 mbar and condenser temperature to -50°C or lower.
X. Dried for 12–24 hours, depending on sample volume and lyoprotectant composition.
XI. Increased the shelf temperature to 20–25°C for 2–4 hours to remove residual moisture, ensuring the final product forms a stable, dry cake.
XII. Sealed the lyophilized samples completely
XIII. Stored at -20°C or 4°C, depending on stability requirements.

POST-LYOPHILIZATION QUANLITY ASSESSMENT

PARAMETER	ACCEPTABLE	MARGINAL	UNACCEPTABLE
Appearance	White, fluffy cake	Slight Collapse	Dark colour, melted
Cake Structure	Uniform, intact	Minor Cracks	Severe collapse
Reconstitution Time	<2 minutes	2-5 minutes	>5 minutes

Table 9 : Visual Inspection Criteria in Physical Characterization Process

DNA INTEGRITY POST-LYOPHILIZATION

I. Added exactly 50 µL nuclease-free water to lyophilized cake.
II. Incubated at room temperature for 5 minutes.
III. Carefully vortexed for a duration of 30 seconds.
IV. Permitted total dissolution (visual confirmation).
V. The analysis was conducted promptly, followed by storage at 4°C.

VI. Spectrophotometric analysis, assessing concentration and purity, alongside agarose gel electrophoresis, evaluating integrity, was conducted.

PLASMID STORAGE

For short-term storage (up to 6 months), purified plasmids were stored in TE buffer at -20°C.

- **For long-term archiving, two complementary methods were employed:**
  - i. **Glycerol Stocks: Overnight bacterial cultures (1 ml) were mixed with sterile 50% glycerol (1 ml)(1:1 ratio) in sealed MCTs and stored at -80°C.**
  - ii. **Purified plasmid DNA (1 µg) was mixed with 10 µL of 20 mM Tris-HCl (pH 8.0) containing 3% trehalose as a cryoprotectant. Samples were lyophilized using a SpeedVac concentrator and stored at -20°C.**

BLOTTING PAPER PRESERVATION OF BACTERIAL AND MAMMALIAN PLASMIDS

Blotting Paper Preservation method relies on the adsorption of DNA onto sterile filter paper, which protects the nucleic acids from degradation while maintaining their viability for transformation experiments. The method is particularly useful for creating backup stocks of valuable plasmid constructs and for sharing plasmids between laboratories.

- Using sterile scissors, we have precisely cut the Trans-Blot papers into rectangular pieces measuring x*y cm^2. Subsequently, circles were drawn on these pieces to create a disc shape for accurately dropping the DNA solution.
- The shaped papers were placed in sterile petri dishes, each containing 5 pieces of papers, and autoclaved at 121°C for 15 minutes at 15 psi. Following autoclaving, the papers were permitted to cool and dry in a sterile setting.
- Prior to utilization, all filter paper pieces were thoroughly dried to guarantee optimal condition and inspected for contamination or any signs of damage.
- All the measures and verified plasmids after the curation procedure were selected for subsequent procedures.
- Using sterile forceps, we placed individual filter paper discs on a sterile surface.
- Each circle was treated with precisely 1 µg of plasmid DNA solution applied to its center.
- Ensured even distribution without oversaturation.
- We allowed complete permeation of DNA into the paper matrix.
- The documents were permitted to dry undisturbed at stable room temperature for a period of 15 to 20 minutes.
- The drying process was conducted in a controlled, dust-free setting, ensuring full drying while carefully avoiding exposure to direct sunlight or excessive heat.
- The cut pieces were securely wrapped in aluminum foil.
- The foil-wrapped blot papers were labeled with the plasmid name, date, concentration, and the amount of DNA contained within.
- Stored at room temperature (20-25°C) under low humidity and dark environment within airtight zip logs.

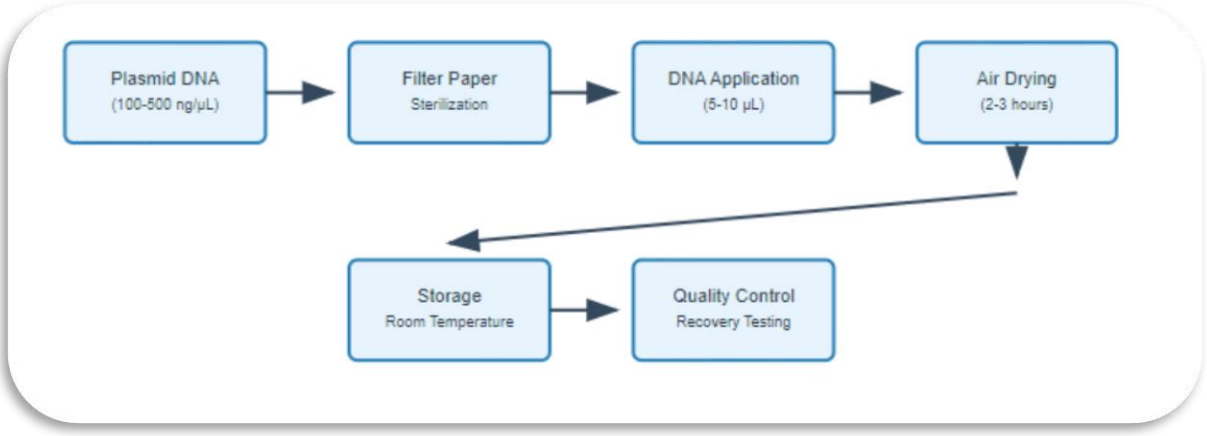


Figure 20 : Schematic Overview of Blotting Paper Preservation Process

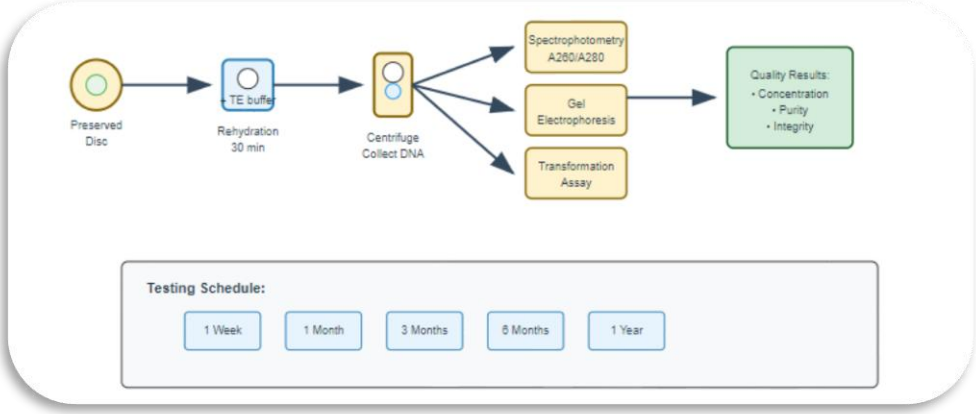


Figure 21 : DNA Recovery and Quality Control Workflow

DIGITAL AND PHYSICAL INDEXING SYSTEM

We have followed the systematic methodology employed for developing and implementing a physical indexing system for curated bacterial and mammalian expression vectors. This approach involves assessing collections, crafting classification frameworks, setting quality control protocols, strategizing physical organization, and integrating digital databases. Through this standardized system, we processed a total of 52 plasmid vectors.

Consumables and Storage Materials

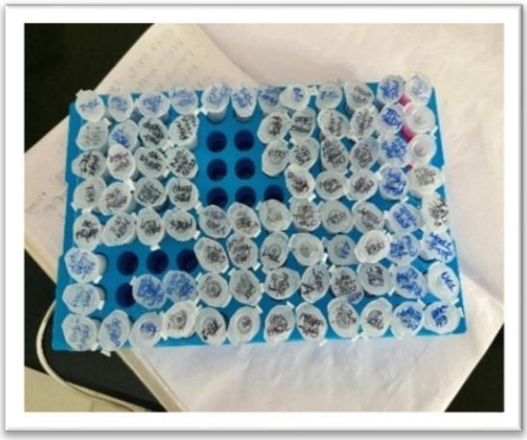
- 1.5 mL microcentrifuge tubes (Eppendorf)
- 96-well storage boxes with alphanumeric grid (Corning)
- Waterproof labels

Organization Hierarchy:

Freezer → Rack → Box → Position → Tube

PLASMID COLLECTION CHARACTERIZATION

Step 1: Physical Count and Documentation



- Removed all plasmid stocks from existing storage
- Conducted manual count of individual tubes
- Recorded preliminary information available on existing labels Photographed each tube for documentation
- Separated vectors by expression system based on visible markings

Grouped by apparent vector series (pET, pcDNA, etc.)

Figure 22: Non-Organized Plasmid Samples; 96 Well Grid Box With 49 Isolated And Purified Plasmids

- Noted any vectors requiring additional characterization.

Total Plasmids (N) = Σ(Bacterial vectors) + Σ(Mammalian vectors)

N = X + Y = 52 plasmids

Step 2: Categorization Calculations:

- **Bacterial vector percentage = (x/52) × 100**
- **Mammalian vector percentage = (y/52) × 100**

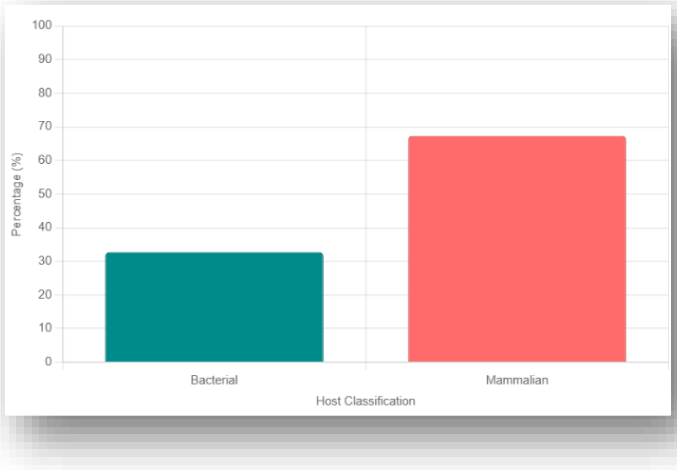


Figure 23 : Distribution of Bacterial and Mammalian Plasmids; Bar graph showing the number of bacterial and mammalian plasmids

Step 3: Detailed Vector Series Identification And Classification

An identification protocol was systematically applied to each plasmid:

- Examined existing labels for vector name and series information
- When necessary, performed restriction digest analysis (We performed hUBCH10-pET29a+)

Distribution calculation methodology:

For each vector series i:

Percentage of series, i = (Count of series i / Total plasmids) × 100

Step 5: Nomenclature Standardization

Standardized Naming Convention Development:

[System Prefix]-[Sequential Number]: [Original Name]

Where: - BAC: Bacterial expression system - MAM: Mammalian expression system - Sequential number: 001-052

- Assigned system prefix based on classification
- Numbered sequentially within each category
- Maintained the original name for reference

Step 6: Physical Organization Protocol And Storage Infrastructure Setup

- **Primary Storage:** -20°C freezer
- **Container:** 2 × 96-well storage boxes
- **Capacity Utilization:** 52/192 positions = 27.08 %
- **Reserved Expansion:** 140 positions available

Box Organization Strategy:

- **Box 1:** BAC-001 to BAC-017 + MAM-001 to MAM-009
- **Box 2:** MAM-010 to MAM-035 + Reserved positions

TABLE 10 : Systematic Classification of Plasmid Stocks

INDEX	PLASMID NAME	CATEGORY	VECTOR SERIES	NOTES FOR FUTURE RESTORATION/ VALIDATION
BAC-001	pGEX-4T1	BACTERIAL	pGEX	Verify supercoiled DNA via gel; test transformation efficacy
BAC-002	TA-4	BACTERIAL	TA	Confirm insert via PCR; test cloning efficiency.

BAC-003	**pGEX-GM3 #3**	**BACTERIAL**	**pGEX**	**Quantify supercoiled DNA via ImageJ; test protein expression.**
BAC-004	**TA-3**	**BACTERIAL**	**TA**	**Verify insert integrity.**
BAC-005	**TA-361BP**	**BACTERIAL**	**TA**	**Confirm insert size via PCR and gel electrophoresis.**
BAC-006	**PGEX-5X-2-GM4**	**BACTERIAL**	**pGEX**	**Quantify supercoiled DNA; test GST-tagged protein expression.**
BAC-007	**UBCH10**	**BACTERIAL**	**pET**	**Test Protein Expression in BL21(DE3) cells**
BAC-008	**U61**	**BACTERIAL**	**Unique/Ambiguous**	**Confirm cloning efficiency; verify insert.**
BAC-009	**PG5E4 IN DH5α**	**BACTERIAL**	**pGEX**	**Confirm insert via PCR; test transformation efficiency.**
BAC-010	**PBS-EMP**	**BACTERIAL**	**pBS**	**Use as cloning control; verify insert absence.**
BAC-011	**TA-1**	**BACTERIAL**	**TA**	**Verify insert integrity.**
BAC-012	**TA-2**	**BACTERIAL**	**TA**	**Verify insert integrity.**
BAC-013	**PSD 4**	**BACTERIAL**	**Unique/Ambiguous**	**Confirm cloning efficiency; verify insert.**
BAC-014	**PJS55-CDC20**	**BACTERIAL**	**Unique/Ambiguous**	**Test CDC20 expression; sequence for integrity**
BAC-015	**ΔN UBCH10**	**BACTERIAL**	**pET**	**Test UBCH10 expression; sequence deletion region.**
BAC-016	**pGEX-5X-2**	**BACTERIAL**	**pGEX**	**Verify GST tag functionality; confirm tac promoter activity in E. coli BL21(DE3); sequence insert for integrity.**
BAC-017	**ΔC pGEX-5X-2**	**BACTERIAL**	**pGEX**	**Check impact of C-terminal deletion on GST fusion protein stability; test expression levels in E. coli BL21(DE3); sequence to confirm deletion boundaries.**

MAM-001	**AUF1-sh(hNRPD-sh LENTI)**	**MAMMALIAN**	**Lentiviral**	**Test transfection efficiency in HEK293 cells; ensure low endotoxin**
MAM-002	**CUEDC2-S110A**	**MAMMALIAN**	**pCMV/pcDNA**	**Validate expression in mammalian cells; sequence for S110A mutation**
MAM-003	**CUEDC2-WT**	**MAMMALIAN**	**pCMV/pcDNA**	**Compare expression with S110A mutant; verify sequence**
MAM-004	**pGL3 181 BP E2 PROMOTER**	**MAMMALIAN**	**pGL3**	**Compare luciferase activity with 733 bp variant**
MAM-005	**pGL3 733 BP E2 PROMOTER**	**MAMMALIAN**	**pGL3**	**Test luciferase activity in mammalian cells; quantify promoter strength**
MAM-006	**CDC20 pTRE-TIGHT**	**MAMMALIAN**	**pTRE-Tight**	**Test Tet-On/Off inducibility; verify CDC20 expression**
MAM-007	**H3-CMV-pFLAG**	**MAMMALIAN**	**pTRE-Tight**	**Validate FLAG-tagged protein expression via Western Blot**
MAM-008	**LYS MUT CDC20**	**MAMMALIAN**	**pCMV/pcDNA**	**Sequence for lysine mutation; test cell cycle effects**
MAM-009	**MAD1-GFP**	**MAMMALIAN**	**pEGFP**	**Assess GFP fluorescence in mammalian cells**
MAM-010	**ΔN- PEGFP**	**MAMMALIAN**	**pEGFP**	**Validate GFP expression; sequence deletion region**
MAM-011	**ΔC-PEGFP**	**MAMMALIAN**	**pEGFP**	**Check expression levels**
MAM-012	**pEGFP-N2 EMPTY VECTOR**	**MAMMALIAN**	**pEGFP**	**Use as a control for pEGFP-based plasmids; test transfection**
MAM-013	**PGL3-361 BP E2 PROMOTER**	**MAMMALIAN**	**pGL3**	**Test luciferase activity; compare with other pGL3 variants**
MAM-014	**PGL3-983 BP**	**MAMMALIAN**	**pGL3**	**Similar to PGL3-361BP; assess promoter strength**
MAM-015	**R4-PEGFP**	**MAMMALIAN**	**pEGFP**	**Validate GFP expression; confirm insert presence**

MAM-016	**RFP-MAD2**	**MAMMALIAN**	**pRFP**	**Assess RFP fluorescence in mammalian cells**
MAM-017	**PTRE-TIGHT EMPTY VECTOR**	**MAMMALIAN**	**pTRE-Tight**	**Use as control for pTRE-TIGHT plasmids; test inducibility**
MAM-018	**pcDNA-NUP**	**MAMMALIAN**	**pcDNA**	**Validate NUP expression; ensure low endotoxin levels**
MAM-019	**APC-5**	**MAMMALIAN**	**pCMV/pcDNA**	**Test expression in mammalian cells; verify sequence**
MAM-020	**E2F1-MUT 361BP UBCH10-PGL3**	**MAMMALIAN**	**pGL3**	**Test luciferase activity; compare with wild-type promoter**
MAM-021	**PTRE-TIGHT LUCIFERASE**	**MAMMALIAN**	**pTRE-Tight**	**Assess the inducibility of the pTRE-Tight Test luciferase in mammalian cell systems.**
MAM-022	**MAD1-3`-UTR MUTATION**	**MAMMALIAN**	**pGL3**	**Assess regulatory effects in mammalian cells**
MAM-023	**pCMV-MYC-NUP**	**MAMMALIAN**	**pCMV**	**Validate MYC-tagged protein expression**
MAM-024	**PEGFP-CDC20**	**MAMMALIAN**	**pEGFP**	**Assess GFP fluorescence; validate CDC20 function**
MAM-025	**PGL3-REV-E2-983BP**	**MAMMALIAN**	**pGL3**	**Test luciferase activity; compare with forward promoter**
MAM-026	**PEDZ-6**	**MAMMALIAN**	**Unique/Ambiguous**	**Test transfection efficiency; ensure low endotoxin.**
MAM-027	**PGL3 BASIC**	**MAMMALIAN**	**pGL3**	**Use as cloning control; verify insert absence.**
MAM-028	**N3-CDC20-GFP**	**MAMMALIAN**	**pEGFP**	**Assess GFP fluorescence; validate CDC20 function**
MAM-029	**PEFGP-GM3**	**MAMMALIAN**	**pEGFP**	**Validate GFP expression; confirm insert**
MAM-030	**pcDNA3.1**	**MAMMALIAN**	**pcDNA**	**Protein Expression**
MAM-031	**APC 7**	**MAMMALIAN**	**pCMV/pcDNA**	**Protein Expression**

MAM-032	**NLS-MUT-FL-CDC20-PEGFP**	**MAMMALIAN**	**pEGFP**	**Fluorescent Tagging validation needed.**
MAM-033	**BAC-A9 (PEB-9)**	**MAMMALIAN**	**PEB-9**	**Test stability in DH10B cells; verify insert size.**
BACV-001	**PACHLT-A EMPTY**	**BACULOVIRUS TRANSFER VECTOR; MAMMALIAN**	**Baculovirus Transfer (pAcHLT)**	**Confirm the integrity of the polyhedrin promoter and MCS by sequencing, as baculovirus transfer vectors may degrade over time, affecting recombination efficiency.**
BACV-021	**PACHLT-A-CDC20**	**BACULOVIRUS TRANSFER VECTOR; MAMMALIAN**	**Baculovirus Transfer (pAcHLT)**	**Validate CDC20 expression; test cell cycle effects.**

Labeling System Implementation:

- **Primary Labels:** Waterproof labels with index code
- **Color Coding:**
  - Blue dots: Bacterial vectors (n= 17)
  - Red dots: Mammalian vectors (n= 35)
- **Position Mapping:** Alphanumeric grid (A1-H12)

Step 7: Quality Control Assessment Protocol

Sample Preparation:

- Thawed plasmid stocks on ice (10 minutes)
- Mixed gently by pipetting
- Aliquoted 2 µL for analysis
- Returned the sample to the stock to store immediately

Figure 24 : Spectrophotometric analysis

Concentration Measurement:

- **Method:** NanoDrop spectrophotometry (wavelength- 260nm) (A260/A280: 1.8-2.0)
- **Blank:** Either TE buffer or nuclease free water.

- **Volume required:** 2-5 µL per sample (DNA Conc- ≥100 ng/µL)

Integrity Verification:

- **Performed 0.8 % agarose gel electrophoresis with Ethidium Bromide staining and 1kb DNA ladder standard.**
- **Samples showing degradation or unusual migration patterns were excluded from analysis.**
- **Inspected under ChemiDoc UV Illuminator and analyzed.**

Figure 25: Gel Inspection and analysis under ChemiDoc. **Figure 26 :** BIO-RAD ChemiDoc

Step 8: Digital Database Integration

Database Schema

- **Index ID (Primary Key)**
- **Plasmid Name**
- **Vector Backbone**
- **Backbone Size**
- **Expression system**
- **Promoter**
- **Selection marker**
- **Concentration (ng/µL)**
- **Storage location**
- **Preparation date**
- **Date Indexed**

- ♦ Date Of Last Use
- ♦ Quality control status (Descriptive Band Pattern Notation, Pattern With Intesity, Band Pattern Type)

Step 9: Data Entry and Validation

- ► Entered basic pieces of information (name, category, series)
- ► Added quality control measurements to the Spreadsheet (Excel, WPS Office)
- ► Assigned the proper storage location
- ► Validated and cross-checked all entries for completeness
- ► Created one backup database

Table 11 : Step 10: Maintenance Protocol

WEEKLY TASKS	MONTHLY TASKS
✓ Verified storage temperatures ✓ Checked label integrity ✓ Update usage logs	✓ Quality spot-checks (random sampling) ✓ Database backup ✓ Inventory reconciliation

Step 11: Quality Control Assessment

To validate the integrity, we first performed Single Enzyme Digestion to see if the digestion is occurring or not. We used **UBCH10 (hUBCH10-PET29)** for assessment.

- ► **Plasmid Details**: hUBCH10-pET29 construct (~6.2 kb total, with 459 bp hUBCH10 insert)
- ► **Antibiotic Selection**: Kanamycin resistance (50 µg/mL) for pET29 vector

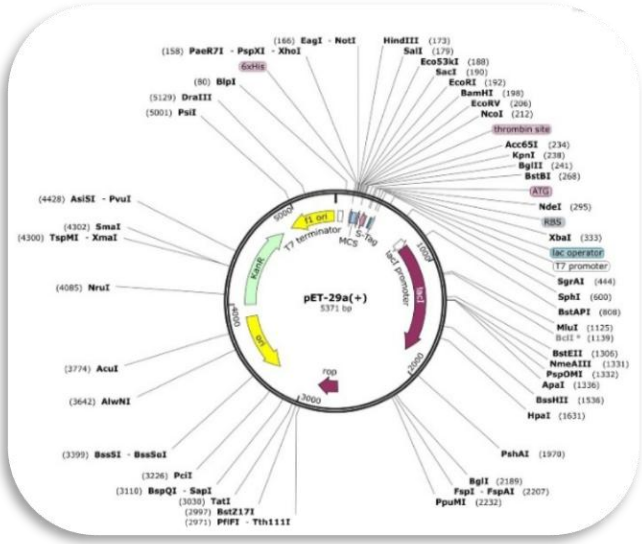


Figure 27: Plasmid Map of pET29a+ vector; taken from SnapGene (Dotmatics)

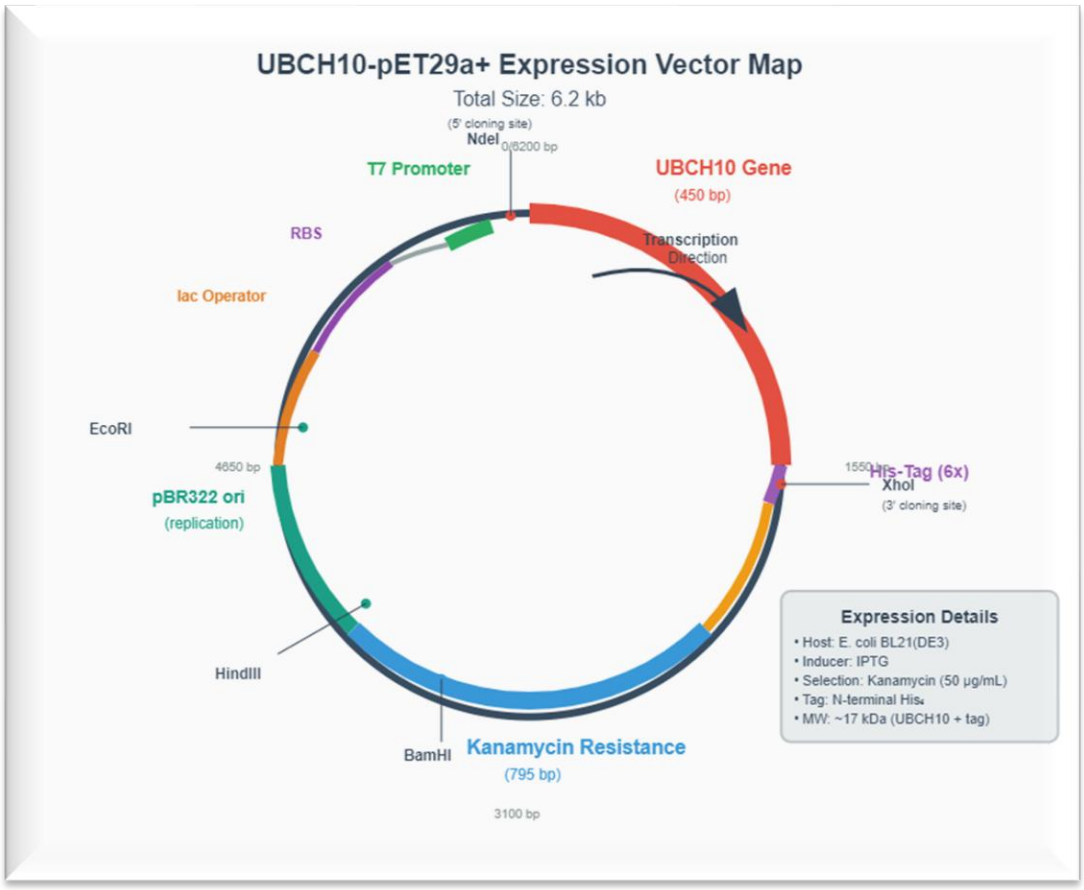


Figure 28: <u>Plasmid Map of the hUBCH10-pET29 construct. Plasmid has a total size of 6.2kb, including a 450 bp insert (hUBCH10). Key features include kanamycin resistance (KanR, 50 µg/mL) for selection in pET Vector, along with annotated restriction sites and functional elements</u> <u>(Biosoft)</u>

- hUBCH10-pET29 plasmid DNA was isolated using GENES2ME SPINXT Plasmid DNA Extraction Kit.
- DNA concentration was measured using BioRad spectrophotometer.
- Purity was assessed by A260/A280 ratio (acceptable range: 1.8-2.0)
- Expected plasmid size: ~ **6.2 kb (hUBCH10 insert ~459 bp + pET29 vector ~5.7 kb)**
- Agarose gel electrophoresis (0.8% gel) was performed to assess plasmid integrity

Figure 29 : GENES2ME SPINXT Plasmid Miniprep Kit

SINGLE ENZYME DIGESTION WITH XhoI

1. **Reaction Setup (50 µL total volume):**
   - ✓ Plasmid DNA: 1 µg
   - ✓ XhoI Restriction enzyme: 1 unit per µg of DNA
   - ✓ rCutSmart Buffer (10×): 5 µL
   - ✓ Sterile water: to final volume of 50 µL
2. **Digestion Conditions:**
   - ✓ Incubation temperature: 37°C
   - ✓ Incubation time: 1-2 hours
   - ✓ Heat inactivation time : 65°C for 20 minutes (optional)
3. **Expected Results:**
   - ► XhoI cuts at single site in pET29a+ multiple cloning site
   - ► Expected linearized plasmid: ~6.2 kb

Figure 30: Restriction Digestion Setup; Setting Water Bath temperature to 37°C

DOUBLE ENZYME DIGESTION WITH EcoRI AND XhoI

Reaction Setup (50 µL total volume):

- ✓ Plasmid DNA: 1 µg
- ✓ XhoI Restriction enzyme: 1 unit per µg of DNA
- ✓ EcoRI Restriction enzyme: 1 unit per µg of DNA
- ✓ TaKaRa Buffer (10×): 5 µL
- ✓ Sterile water: to final volume of 50 µL

Figure 31: Restriction Buffer (TaKaRa)

Figure 32: Restriction Enzymes- XhoI & EcoRI (TaKaRa)

Expected Results:

- EcoRI cuts upstream of hUBCH10 insert
- XhoI cuts downstream of hUBCH10 insert
- Expected fragments:
  i. hUBCH10 insert: ~459 bp
  ii. pET29 vector backbone: ~5.7 kb

GEL ELECTROPHORESIS AND GEL ANALYSIS

- We prepared 1.5 % agarose gel with 1× TAE buffer.
- We added EtBr for visualization
- We used 1kb molecular weight markers.
- **Electrophoresis Conditions:**
  a. **Voltage: 100V**
  b. **Running time: 45-60 minutes**
  c. **Buffer: 1× TAE**

Figure 33: 1kb DNA Ladder (RM30291)

RESULTS AND DISCUSSION

An evaluation was conducted on the effectiveness of different storage methods for plasmid restoration in order to enhance the protocols for managing the plasmid repository. A comprehensive restoration process was executed on 17 bacterial and 35 mammalian expression plasmids, sourced from both glycerol stocks and lyophilized preparations, which had been preserved for a period ranging from 2-15 years.

PLASMID INVENTORY AND CLASSIFICATION

Table 12 : Percentage Distribution of Bacterial and Mammalian Plasmids

CATEGORY	NUMBER OF PLASMIDS	PERCENTAGE(%)
BACTERIAL	17	32.69
MAMMALIAN	35	67.30

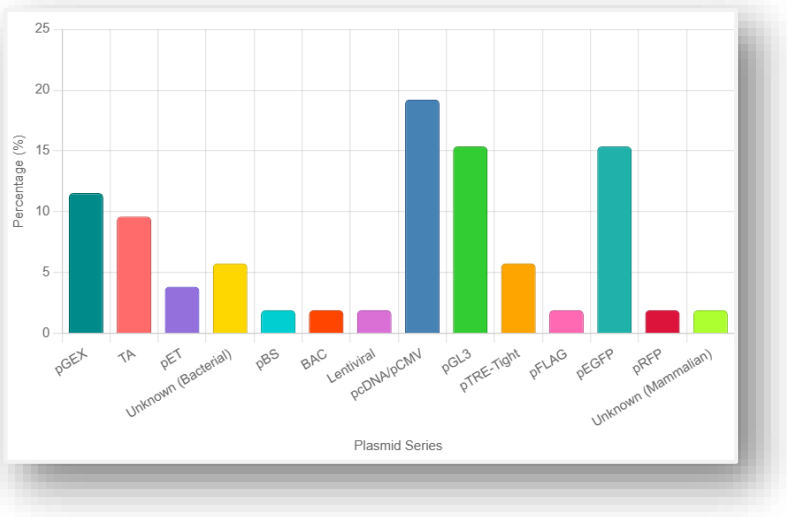
Table 13 : Summary of Plasmid Collection

PLASMID TYPE (CLONED GENE NAME)	NUMBER OF SAMPLES	EXPRESSION SYSTEM	STORAGE CONDITION
pGEX Series	**6**	**tac promoter**	-80°C glycerol stocks
pET Series	**2**	**T7 promoter**	-80°C glycerol stocks
pEGFP Series	**8**	**CMV**	-80°C glycerol stocks
pTRE-TIGHT Series	**3**	**TRE-Tight**	-80°C glycerol stocks
TA Series	**5**	**N/A (lac for resistance)**	-80°C glycerol stocks
pCMV/pcDNA Series	**10**	**CMV**	-80°C glycerol stocks
PBS Series	**1**	**N/A (lac for resistance)**	-80°C glycerol stocks
BAC Series	**1**	**N/A**	-80°C glycerol stocks
Lentiviral Series	**1**	**U6 (for shRNA)**	-80°C glycerol stocks
pGL3 Series	**8**	**E2 (PGL3 BASIC: None)**	-80°C glycerol stocks
pFLAG, pRFP Series	**2**	**CMV**	-80°C glycerol stocks

Unique or ambiguous vectors (Bacterial & Mammalian)	**4**	**Unknown (likely lac or T7), Unknown (likely CMV)**	-80°C glycerol stocks

PERCENTAGE DISTRIBUTION OF PLASMID SERIES

Figure 34 : This Bar graph visualizes the percentage of plasmids in each series (e.g., pGEX, pGL3), as calculated previously: (pGEX: 11.54% , TA: 9.62% ,pET: 3.85% ,Unknown (Bacterial): 5.77% ,pBS: 1.92% ,BAC: 1.92% ,Lentiviral: 1.92% ,pcDNA/pCMV: 19.23% ,pGL3: 15.38%, pTRE-Tight: 5.77% ,pFLAG: 1.92% ,pEGFP: 15.38% ,pRFP: 1.92% ,Unknown (Mammalian): 1.92%)



PLASMID DNA YIELD AND PURITY ASSESSMENT

Plasmid DNA was extracted using the standard alkaline lysis method. Yield and purity were assessed spectrophotometrically.

Table 14 : Plasmid DNA Extraction Results

SAMPLE/ INDEX ID	PLASMID NAME	DNA CONC. (ng/µL)	A260/A280	A260/A230	Total Yield (µg)
BAC-001	**pGEX-4T1**	406.9	1.83	2.29	16.27
BAC-002	**TA-4**	202.7	1.85	2.49	8.108
BAC-003	**pGEX-GM3 #3**	737.4	1.87	2.23	29.49
BAC-004	**TA-3**	686.7	1.93	2.18	27.46
BAC-005	**TA-361BP**	224.3	1.80	1.74	6.72
BAC-006	**PGEX-5X-2-GM4**	412.3	1.78	1.46	16.49
BAC-007	**UBCH10**	365.5	1.89	2.44	14.62

BAC-008	**U61**	1463.1	1.78	2.01	58.524
BAC-009	**PG5E4 IN DH5α**	1445.8	1.79	2.24	57.93
BAC-010	**PBS-EMP**	473.4	1.84	2.40	23.67
BAC-011	**TA-1**	512.9	1.95	2.55	20.51
BAC-012	**TA-2**	1202.0	1.95	2.54	48.08
BAC-013	**PSD4**	103.9	1.84	2.37	3.11
BAC-014	**PJS55-CDC20**	1463.1	1.81	2.19	190.20
BAC-015	**ΔN UBCH10**	104.3	1.79	2.37	3.12
BAC-016	**pGEX-5X-2**	1311.2	1.99	2.56	91.78
BAC-017	**ΔC pGEX-5X-2**	1446.8	2.02	2.49	144.68
MAM-001	**AUF1-sh(hNRPD-sh LENTI)**	293.4	1.78	2.21	14.67
MAM-002	**CUEDC2-S110A**	309.9	1.97	2.43	12.39
MAM-003	**CUEDC2-WT**	1195.6	1.91	2.28	83.69
MAM-004	**pGL3 181 BP E2 PROMOTER**	202.7	2.04	2.38	8.10
MAM-005	**pGL3 733 BP E2 PROMOTER**	269.3	2.00	2.27	13.46
MAM-006	**CDC20 pTRE-TIGHT**	397.5	1.93	2.29	15.90
MAM-007	**H3-CMV-pFLAG**	695.8	1.89	2.29	27.83
MAM-008	**LYS MUT CDC20**	502.2	1.97	2.36	20.08
MAM-009	**MAD1-GFP**	497.7	1.80	1.47	19.90
MAM-010	**ΔN-PEGFP**	1077.2	1.95	1.58	75.40
MAM-011	**ΔC-PEGFP**	276.4	1.79	2.03	8.29
MAM-012	**pEGFP-N2 EMPTY VECTOR**	173.4	1.75	1.36	5.20
MAM-013	**PGL3-361 BP E2 PROMOTER**	223.3	1.84	1.75	6.69

MAM-014	**PGL3-983BP E2 PROMOTER**	412.3	1.76	1.48	16.49
MAM-015	**R4-PEGFP**	80.5	1.85	1.74	3.22
MAM-016	**RFP-MAD2**	237.1	1.86	1.78	7.11
MAM-017	**PTRE-TIGHT EMPTY VECTOR**	88.3	1.79	1.65	3.53
MAM-018	**pcDNA-NUP**	788.0	1.88	2.16	31.52
MAM-019	**APC-5**	949.3	1.93	2.46	66.45
MAM-020	**E2F1-MUT 361BP UBCH10-PGL3**	1121.2	1.89	2.44	44.84
MAM-021	**PTRE-TIGHT LUCIFERASE**	725.5	2.08	2.09	29.02
MAM-022	**MAD1-3`-UTR MUTATION**	1384.4	1.89	2.50	55.37
MAM-023	**pCMV-MYC-NUP**	1247.2	1.97	2.56	49.88
MAM-024	**pEGFP-CDC20**	1272.1	2.04	2.52	87.30
MAM-025	**PGL3-REV-E2-983BP**	1269.1	1.97	2.45	50.76
MAM-026	**PEDZ-6**	397.7	1.98	2.34	11.93
MAM-027	**PGL3 BASIC**	1041.8	1.88	2.28	41.67
MAM-028	**N3-CDC20-GFP**	564.3	1.92	2.50	16.92
MAM-029	**PEFGP-GM3**	641.8	1.96	2.44	25.67
MAM-030	**pcDNA3.1**	930.0	1.88	2.34	37.20
MAM-031	**APC 7**	157.0	1.79	2.41	4.71
MAM-032	**BAC-A9 (PEB-9)**	1329.5	1.75	1.75	53.18
MAM-033	**NLS MUT FL CDC20 PEGFP**	372.7	1.85	2.11	11.18
MAM-033/ BACV-001	**PACHLT-A EMPTY**	734.0	1.82	2.31	29.36

MAM-034/BACV-002	**PACHLT-A-CDC20**	1232.4	1.95	2.51	49.29

-

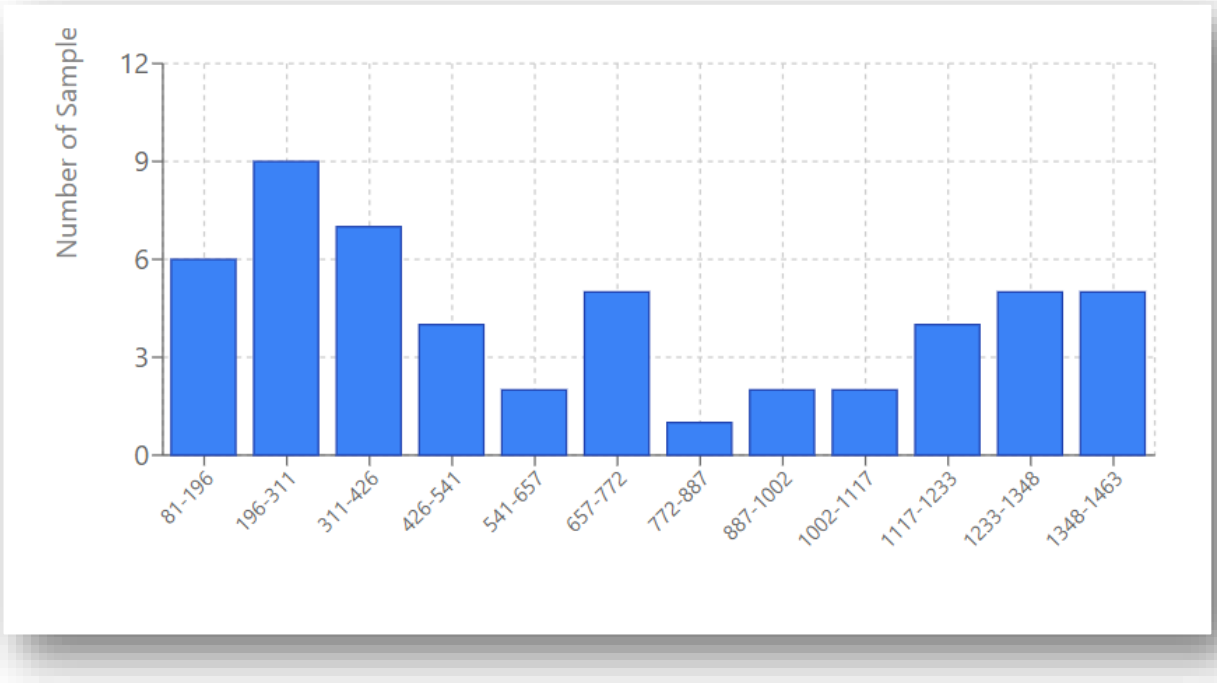


Figure 35 : DNA Concentration Distribution Bar Chart *showing distribution of DNA concentrations across all samples, **X-axis:** DNA concentration ranges (ng/µL) and **Y-axis:** Number of samples in each concentration range; The distribution shows that most plasmid samples have concentrations between 200-800 ng/µL, with a smaller group of high-concentration samples (1200-1500 ng/µL)*

- **Total samples: 52**
- **Mean concentration: 734.5 ng/µL**
- **Median concentration: 489.9 ng/µL**
- **Range: 80.5 - 1463.1 ng/Ml**

Key Observations:

1. The bimodal distribution is distinctly observable, characterized by two separate peaks.
2. **Primary cluster**: ~33 samples between 80-800 ng/µL (lower concentration group)
3. **Secondary cluster**: ~12 samples between 1000-1500 ng/µL (high-yield group)
4. **Gap between clusters**: Very few samples in the 800-1000 ng/µL range

Sample Quality Assessment:

- **Excellent yields (>1000 ng/µL):** 12 samples (23%)
- **Good yields (400-1000 ng/µL):** 18 samples (35%)
- **Moderate yields (200-400 ng/µL):** 16 samples (31%)

- **Low yields (<200 ng/µL):** 6 samples (11%)

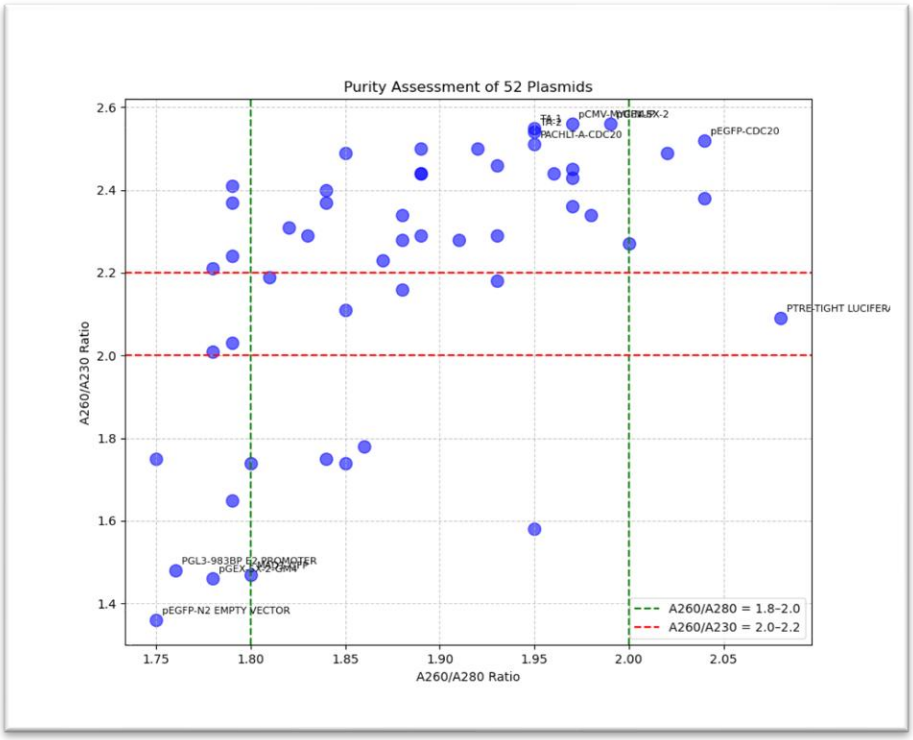


Figure 36 : Purity Assessment *; scatter plot with A260/A280 ratios on X-axis and A260/A230 ratios on Y-axis. Scatter plot of A260/A280 vs. A260/A230 ratios for 52 plasmids. Green dashed lines indicate the acceptable A260/A280 range (1.8–2.0), and red dashed lines indicate the acceptable A260/A230 range (2.0–2.2). Outliers (e.g., pEGFP-N2 EMPTY VECTOR, PTRE-TIGHT LUCIFERASE) are annotated.*

AGAROSE GEL ELECTROPHORESIS ANALYSIS

All isolated plasmids were subjected to agarose gel electrophoresis (0.8-1% agarose in TAE buffer) to verify structural integrity.

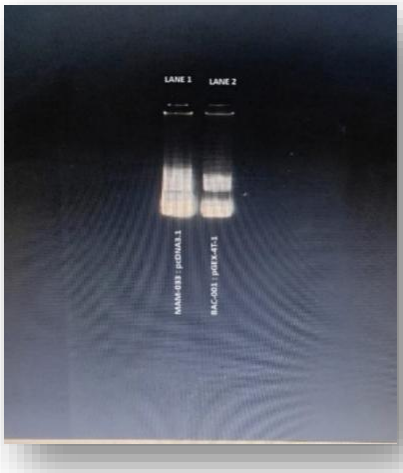
FIGURE 37: Agarose gel electrophoresis confirming structural integrity of isolated plasmids MAM-033 and BAC-001, both displaying characteristic double band patterns. ➲ **MAM-033** DOUBLE BAND PATTERN: Two bands of similar intensity in upper half of gel ➲ **BAC-001** (DOUBLE BAND PATTERN: Two bands of similar intensity in upper half of gel (Doublet /Twin Bands), shortly separated)	

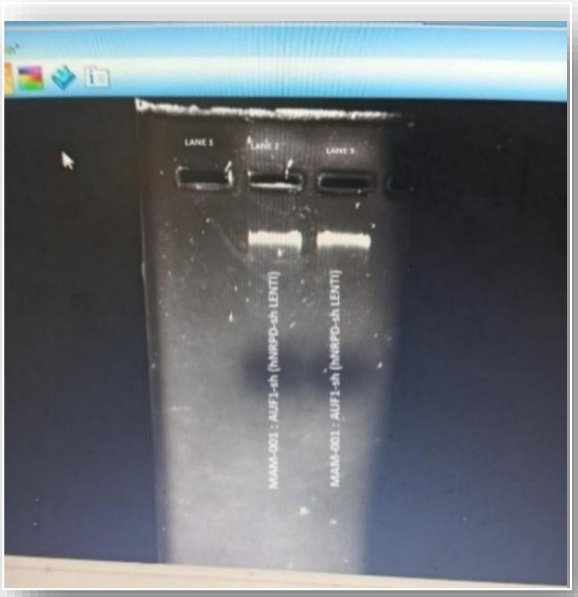
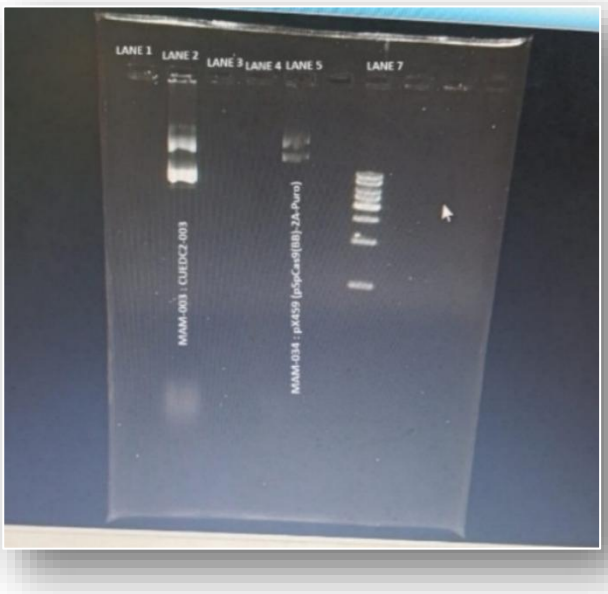
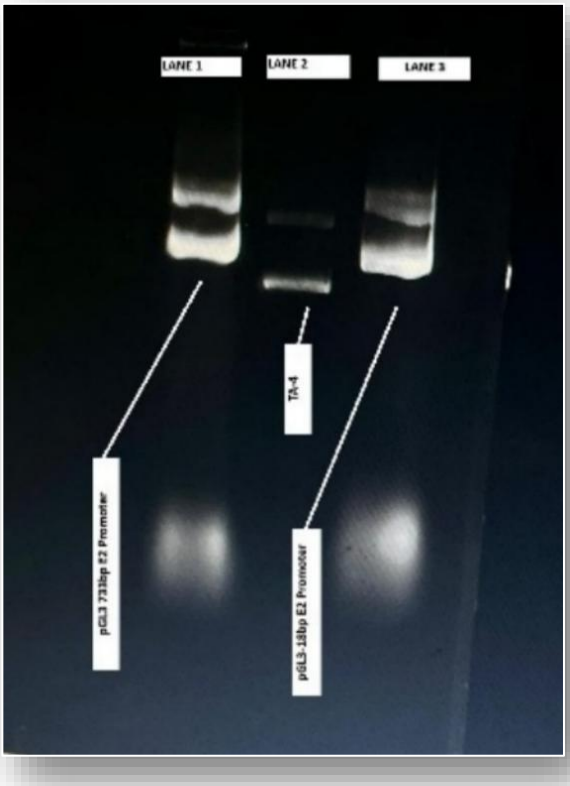
FIGURE 38: <u>Gel electrophoresis analysis of MAM-001 plasmid displaying characteristic single band in upper gel region</u> ➲ **<u>MAM-001</u>** (SINGLE BAND: One sharp band in upper half of gel)	
FIGURE 39<u>: Agarose gel electrophoresis of plasmid MAM-003 showing double band pattern with widely separated bands of similar intensity.</u> ➲ **MAM-003** (DOUBLE BAND PATTERN: Two similar band of same intensity and width in upper half of gel, widely separated)	

FIGURE 40: Gel electrophoresis analysis of BAC-003 displaying complex band pattern with one high intensity band and two lower intensity bands

- **BAC-003**

  (COMPLEX BAND PATTERN: One high intensity band with other two low intensity bands, shortly separated, sort of smears are visible, RNA contamination is present)

FIGURE 41: Comparative gel electrophoresis of plasmids MAM-005, BAC-007, and MAM-004 showing distinct double band patterns for each construct.



- **MAM-005**

  (DOUBLE BAND PATTERN: Two bands of similar intensity in upper half of gel, shortly separated, RNA Contamination at hand)

- **BAC-007**

  (DOUBLE BAND PATTERN: Two bands of different intensities in upper half of gel; one comparatively more intense than the other, widely separated)

- **MAM-004**

  (DOUBLE BAND PATTERN: More or less of similar intensity in the upper half of gel, strictly separated, sort of RNA contamination is there)

FIGURE 42: <u>Gel electrophoresis of plasmids MAM-006, MAM-007, and MAM-008 demonstrating varied banding patterns from complex to double band configurations</u>

➲ **MAM-006**

(COMPLEX PATTERN: Faint smeared high MW band with two distinct lower bands)

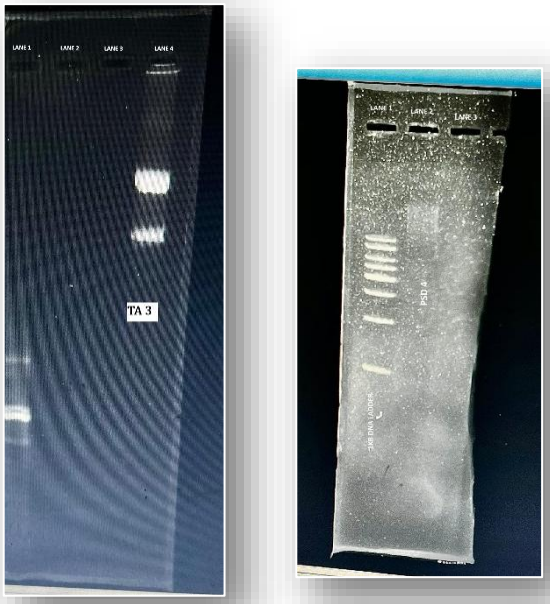
➲ **MAM-007**

(COMPLEX PATTERN: Proper One high MW band with other three distinct lower MW bands)

➲ **MAM-008**

(DOUBLE BAND PATTERN: Two bands of similar intensity, precisely separated)

FIGURE 43: <u>Agarose gel electrophoresis of BAC-004 and BAC-013 showing double and single band patterns with different molecular weight distributions.</u>



➲ **BAC-004**

(DOUBLE BAND PATTERN: Two bands of different intensity and molecular weights respectively, high MW band is following the latter)

➲ **BAC- 013**

Single Band Pattern: One faint upside low density band has appeared

FIGURE 44: Agarose gel electrophoresis showing DNA band patterns for samples BAC-005, MAM-010, BAC-013, MAM-011, BAC-006, MAM-012, MAM-013, and MAM-014. Multiple discrete bands of varying molecular weights are visible across lanes.

➲ **BAC-005**

(TRIPPLE BAND PATTERN: High MW band is in the last with two lower MW bands leading, smear is noticeable)

➲ **MAM-010**

SINGLE BAND: One distinct band (low width) is evident (~top 1/4 of gel)

➲ **BAC-012**

(Thin one band is apparent)

➲ **MAM-011**

(Smeared appeareance along the lane)

➲ **BAC-006**

SINGLE BAND: Neat band (high MW) is evident (~top 1/4 of gel)

➲ **MAM-012**

SINGLE BAND: Neat band (high MW) is apparent (~top 1/4 of gel)

➲ **MAM-013**

DOUBLE BAND PATTERN: One clear band of high MW is prominent with one partly segregated low MW band (noticeable)

➲ **MAM-014**

DOUBLE BAND PATTERN: Neat band of high MW is prominent with one partly pulled low MW band

FIGURE 45: Gel electrophoresis results displaying samples MAM-015 (double band pattern with higher and lower intensity bands), MAM-016 (double band pattern with different intensities), and MAM-017 (single sharp band in mid-lane position).

➲ **MAM-015**

DOUBLE BAND PATTERN: One band with higher intensity with a lower intensity higher MW band

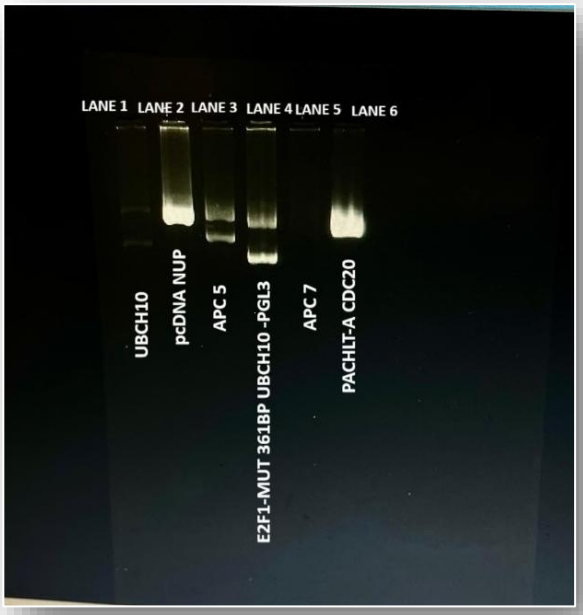
➲ **MAM-016**

DOUBLE BAND PATTERN: Two different bands with different intensities

➲ **MAM-017**

SINGLE BAND: One sharp band evident in the mid-way of lane

FIGURE 46: **Six-lane electrophoresis gel displaying samples BAC-007 (double band pattern), MAM-018 (single elevated band), MAM-019 (double band pattern with nearly similar bands), MAM-020 (double band pattern with indifferent intensities), MAM-031 (empty lane), and MAM-034/BACV-002 (single high-intensity band with evident contamination)**



➲ **BAC-007**

DOUBLE BAND PATTERN: Two bands of similar intensity, ill-defined

➲ **MAM-018**

SINGLE BAND apparent with elevated acuteness(high MW)

➲ **MAM-019**

DOUBLE BAND PATTERN: Two nearly similar bands in size with minimal different intensities are prominent, separation is straightforward

➲ **MAM-020**

DOUBLE BAND PATTERN: Two nearly similar bands in size with quite indifferent intensities are prominent, broadly separated

➲ **MAM-031**

Lane is empty

➲ **MAM-034/BACV-002**

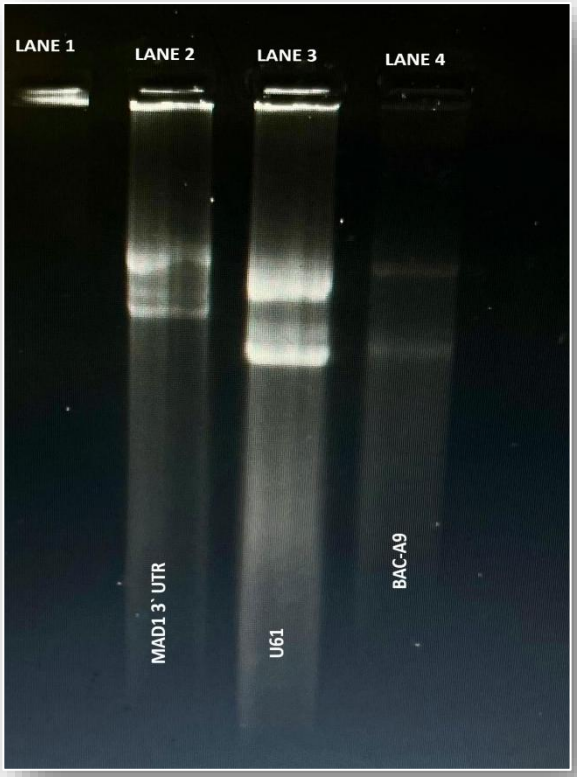
Single High Intensity Band with evident MW, separation is very lucid, no contamination is anticipated so far

FIGURE 47: Electrophoresis gel showing sample MAM-021 with two discrete bands of modest intensity, demonstrating tangible band transparency.

➲ **MAM-021**

Two discrete bands with modest intensity, transparency is tangible

FIGURE 48: <u>Multi-lane gel analysis showing samples MAM-022 (triple band pattern with fragile, modest intensity bands), BAC-008 (two similar bands with analogous intensities), and MAM-032 (two feeble bands with obtained smears).</u>



➲ **MAM-022**

TRIPPLE BAND PATTERN: Relatively fragile, modest intensity and band strength is little compromised in middle band , smudges are emerging

➲ **BAC-008**

TWO SIMILAR BANDS with analogously similar band width and intensity, separation is exact, smears are marked

➲ **MAM-032**

Two feeble bands(same MW) are obtained, smears are intense

FIGURE 49: <u>Lane analysis reveals BAC-012 with triple band pattern indicating high MW bands, MAM-001 displaying single band pattern, MAM-023 showing empty lane, and BAC-014 exhibiting double band pattern with discrete high MW bands.</u>

➲ **BAC-012**

TRIPLE BAND PATTERN: One intense moderate MW band(~top 1/4 of gel), two other with nearly similar width, unequally distributed bands are residing underneath, smears are evident

➲ **MAM-001**

Single Band evident

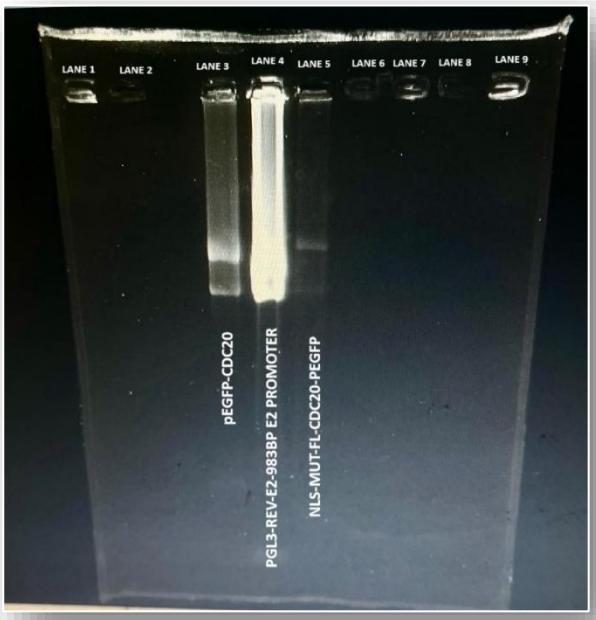
➲ **MAM-023**

Lane is empty

➲ **BAC-014**

DOUBLE BAND PATTERN: Two discrete fierce (high MW) bands are detectable with massive smear content

FIGURE 50: **Electrophoretic analysis of restored plasmids demonstrating varied banding patterns. MAM-024 shows double band pattern with different sized bands, MAM-025 exhibits complex smearing pattern with indistinct bands, and MAM-033 displays double bands of different molecular weights.**



➲ **MAM-024**

DOUBLE BAND PATTERN: Two different bands(one is high MW,other is lower MW), distribution is proper, smears are somewhat extant

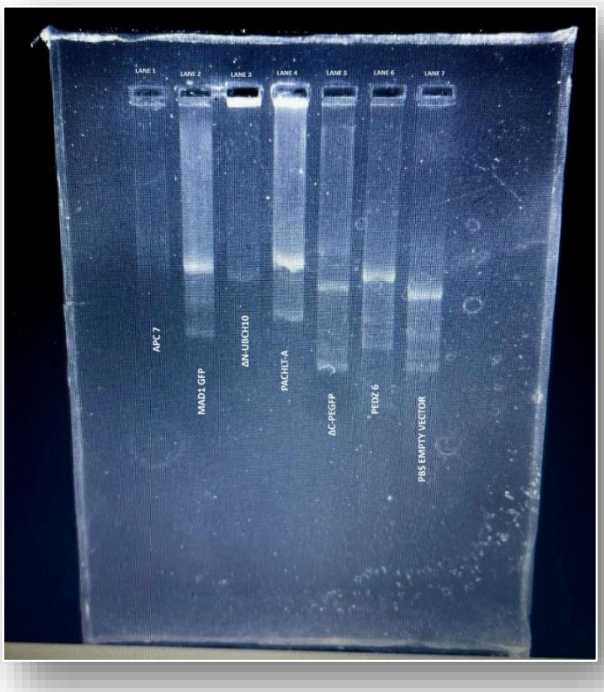
➲ **MAM-025**

Extensive smeared lane, COMPLEX PATTERN: Thick intense band is leading, other two most indistinct bands remaining in the middle and last moderate one runnning late

➲ **MAM-033**

DOUBLE BAND: Bands are muzzy and are of different sizes (MW)

FIGURE 51: Multiple samples (MAM-031, MAM-009, BAC-015, MAM-033/BACV-001, MAM-011, MAM-026, BAC-010) demonstrate varying band intensities and patterns, indicating different levels of DNA recovery and integrity.



➲ **MAM-031**

Degraded DNA along the lane, intensity is also compromised

➲ **MAM-009**

TRIPPLE BAND PATTERN: High MW band is in the last with two lower MW bands leading, smear is instant

➲ **BAC-015**

Slight Single Faint band is visible, huge smear seizure

➲ **MAM-033/BACV-001**

DOUBLE BAND PATTERN: ONE BAND IS OF HIGHER INTENSITY, Integrity is endangered

➲ **MAM-011**

COMPLEX BAND PATTERN: Three feeble bands clearly evident, least distinct one is running after the leader band(similarly alike)

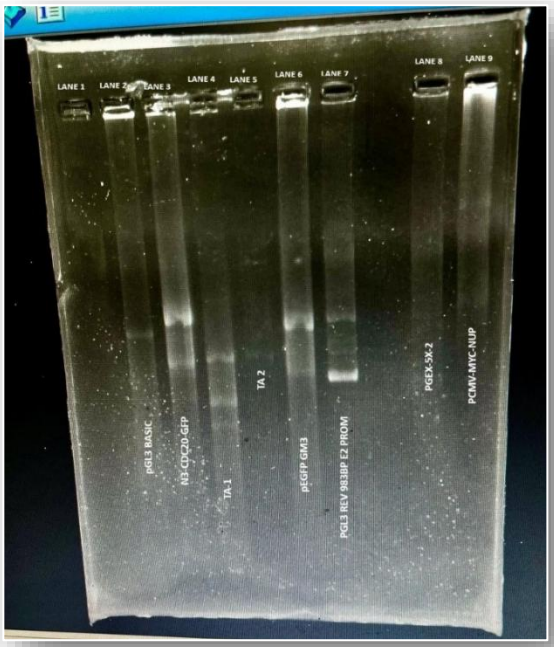
➲ **MAM-026**

COMPLEX PATTERN: Smeared high MW band with three imperceptible bands of lower MWs

➲ **BAC-010**

COMPLEX PATTERN: Smeared high MW band (crisp) with three nearly obscure bands of lower MWs

FIGURE 52: Samples MAM-027 through MAM-023 exhibit varied electrophoretic profiles including single low MW bands, discrete separated bands, thick intense bands, and complex smearing patterns, demonstrating successful DNA recovery with different molecular weight distributions.



- **MAM- 027**

Very obscure SINGLE LOW MW BAND with significant smears along the lane, smears are apparent

- **MAM-028**

Two discrete, fatigued non-similar(MW) bands, smears are proper, widely spread

- **BAC-011**

Three distinct (almost similar MW) vague bands, separated clearly(one at a distance, other twos are aligning nearby)

- **BAC-012**

Thin one band is apparent

- **MAM-029**

DOUBLE BOND PATTERN: One thick intense band & other is too obscure relatively with lower MW, smears are clear

- **MAM-025**

COMPLEX PATTERN: Thick intense band is leading, other two most indistinct bands remaining in the middle and last moderate one runnning late

- **BAC-016**

Smeared pattern, no distinct band visible

- **MAM-023**

No bands were visible, lane is smeared

FIGURE 53: BAC-004 displays single band pattern with moderate intensity and visible smearing, BAC-017 exhibits triple band pattern with one intact high MW band and two partially separated bands of different intensities, and MAM-010 shows two distinct bands with similar intensity and available smears.

- **BAC-004**

Single Band pattern, moderate intensity, Smears are present

- **BAC-017**

TRIPPLE BAND PATTERN: One intact low MW band is ahead of two partially separated bands of different intensities.

- **MAM-010**

Two distinct bands with similar intensity, smears available

Table 15 : Gel Electrophoresis Results Summary

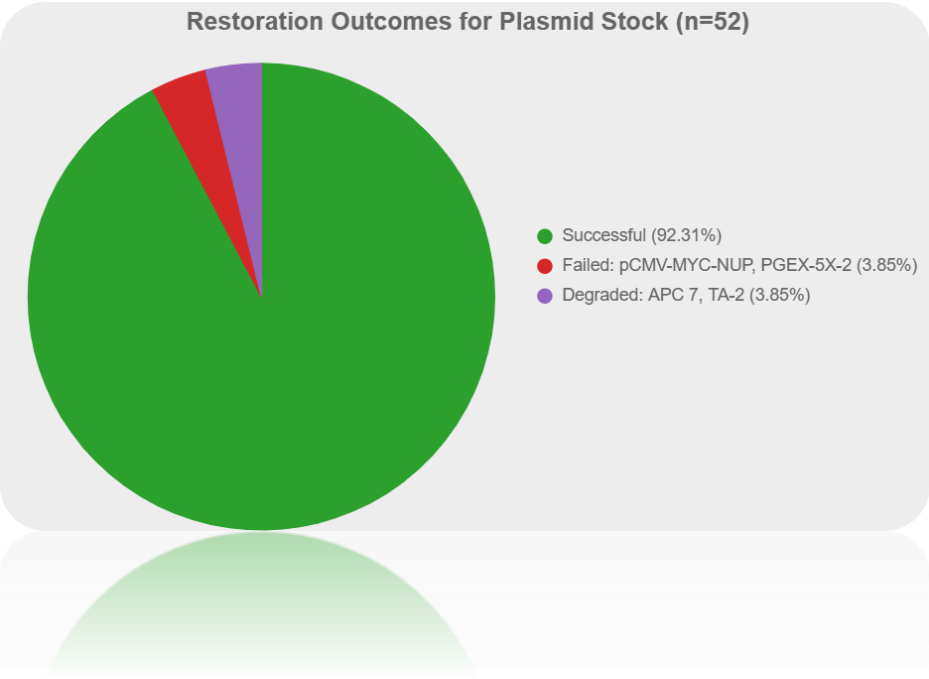
SAMPLE ID	BAND QUALITY	BAND INTENSITY (1= FAINT, 5= INTENSE)	INTEGRITY STATUS
BAC-001	**pGEX-4T1**	HIGH MW- 5,MEDIUM MW- 4	**GOOD**
BAC-002	**TA-4**	HIGH MW- 2, LOW MW- 4	**GOOD**
BAC-003	**pGEX-GM3 #3**	HIGH MW- 3, LOWER MW- 1,1	**MODERATE**
BAC-004	**TA-3**	HIGH MW- 5, LOW MW- 4	**GOOD**
BAC-005	**TA-361BP**	HIGH MW- 4, LOWER MWs- 3,3	**GOOD**
BAC-006	**PGEX-5X-2-GM4**	SINGLE UP-RIGHT POSITIONED BAND- 5	**GOOD**
BAC-007	**UBCH10**	HIGH MW- 2, LOW MW-2	**MODERATE**
BAC-008	**U61**	HIGH MW- 4, LOW MW-4	**GOOD**
BAC-009	**PG5E4 IN DH5α**	HIGH MW-4, LOWER MWs- 3,4	**GOOD**
BAC-010	**PBS-EMP**	HIGH MW-3, LOWER MWs-1,1,1	**MODERATE**
BAC-011	**TA-1**	HIGH MW-2,MEDIUM MW-2, LOW MW-1	**POOR TO MODERATE**
BAC-012	**TA-2**	LOW MW- 2	**POOR**
BAC-013	**PSD4**	SINGLE MIDWAY BAND- 3	**POOR**
BAC-014	**PJS55-CDC20**	HIGH MW- 5, LOW MW-4	**GOOD**
BAC-015	**ΔN UBCH10**	SINGLE UP-RIGHT POSITIONED BAND- 1	**POOR**
BAC-016	**pGEX-5X-2**	N/A	**DEGRADED**
BAC-017	**ΔC pGEX-5X-2**	HIGH MW- 2,1; LOW MW- 3	**MODERATE**
MAM-001	**AUF1-sh(hNRPD-sh LENTI)**	SINGLE UPRIGHT-POSITIONED BAND- 4	**GOOD**
MAM-002	**CUEDC2-S110A**	HIGH MW- 5, MEDIUM MW- 3	**GOOD**
MAM-003	**CUEDC2-WT**	HIGH MW- 5, LOWER MW- 3	**GOOD**
MAM-004	**pGL3 181 BP E2 PROMOTER**	HIGH MW-3, LOW MW-4	**GOOD**

MAM-005	**pGL3 733 BP E2 PROMOTER**	HIGH MW-3, LOW MW- 4	**GOOD**
MAM-006	**CDC20 pTRE-TIGHT**	HIGH MW- 2, LOWER MWs- 3,4	**MODERATE**
MAM-007	**H3-CMV-pFLAG**	HIGH MW-3, LOWER MWs- 3,3,4	**GOOD**
MAM-008	**LYS MUT CDC20**	HIGH MW-2, LOW MW- 3	**MODERATE**
MAM-009	**MAD1-GFP**	HIGH MW- 4, MEDIUM AND LOW MW-1,1	**POOR TO MODERATE**
MAM-010	**ΔN-PEGFP**	SINGLE UPRIGHT-POSITIONED BAND- 4	**MODERATE**
MAM-011	**ΔC-PEGFP**	HIGH MW- 1, MODERATE MW- 2, LOW MW-2	**POOR**
MAM-012	**pEGFP-N2 EMPTY VECTOR**	SINGLE UP-RIGHTPOSITIONED BAND- 5	**GOOD**
MAM-013	**PGL3-361 BP E2 PROMOTER**	HIGH MW- 5, LOW MW-2	**GOOD**
MAM-014	**PGL3-983BP E2 PROMOTER**	HIGH MW- 5, LOW MW-2	**GOOD**
MAM-015	**R4-PEGFP**	HIGH MW- 2, LOW MW-4	**MODERATE**
MAM-016	**RFP-MAD2**	HIGH MW- 3, LOW MW-4	**MODERATE TO GOOD**
MAM-017	**PTRE-TIGHT EMPTY VECTOR**	SINGLE UP-RIGHT POSITIONED BAND-4	**MODERATE**
MAM-018	**pcDNA-NUP**	SINGLE UPRIGHT-POSITIONED BAND- 5	**GOOD**
MAM-019	**APC-5**	HIGH MW- 3, LOW MW- 3	**POOR**
MAM-020	**E2F1-MUT 361BP UBCH10-PGL3**	HIGH MW- 3, LOW MW- 4	**MODERATE**
MAM-021	**PTRE-TIGHT LUCIFERASE**	HIGH MW- 3, LOW MW-4	**POOR TO MODERATE**
MAM-022	**MAD1-3`-UTR MUTATION**	HIGH MW-3, MODERATE MW-1, LOW MW-2	**MODERATE**
MAM-023	**pCMV-MYC-NUP**	N/A	**DEGRADED**
MAM-024	**pEGFP-CDC20**	HIGH MW-4, LOW MW-3	**MODERATE**

MAM-025	**PGL3-REV-E2-983BP**	HIGH MW- 2 MODERATE MW- 1, LOW MW- 3	**POOR TO MODERATE**
MAM-026	**PEDZ-6**	HIGH MW-3, LOWER MWs-1,1,1	**POOR**
MAM-027	**PGL3 BASIC**	SINGLE MID-WAY BAND- 1.5	**POOR**
MAM-028	**N3-CDC20-GFP**	HIGH MW- 3.5, LOW MW-2	**POOR**
MAM-029	**PEFGP-GM3**	HIGH MW-3, LOW MW-1.7	**POOR**
MAM-030	**pcDNA3.1**	HIGH MW- 4, LOWER MW-4	**GOOD**
MAM-031	**APC 7**	High MW- 1-2, Low MW- 1-2	**DEGRADED/ PRONE TO DEGRADATION**
MAM-032	**BAC-A9 (PEB-9)**	HIGH MW-2, LOW MW-2	**POOR**
MAM-033	**NLS MUT FL CDC20 PEGFP**	HIGH MW-2, LOW MW-1	**POOR TO MODERATE**
MAM-034/ BACV-001	**PACHLT-A EMPTY**	HIGH MW- 2, LOW MW- 3	**POOR TO MODERATE**
MAM-035/BACV-002	**PACHLT-A-CDC20**	SINGLE UP-RIGHT POSITIONED BAND-5	**GOOD**

SUCCESS RATE ANALYSIS

Figure 54 : Plasmid Recovery Success Rate ; Pie Chart showing percentages of successful isolations, failed isolations, and degraded samples



POST-LYOPHILIZATION QUALITY ASSESSMENT

Table 16 : Post-Lyophilization Quality Assessment of Restored Plasmid Collection : This table presents the comprehensive post-lyophilization quality assessment results for all 52 restored plasmid samples. Each sample was evaluated based on final DNA concentration (ng/µL), total yield obtained after isolation and purification (µg), visual appearance characteristics, physical cake structure integrity, and overall quality classification. Assessment criteria were established based on concentration thresholds: samples >200 ng/µL classified as acceptable (white, fluffy cake with uniform structure), 80-200 ng/µL as marginal (slight collapse with minor cracks), and <80 ng/µL as unacceptable (dark color with severe structural collapse).

SAMPLE ID	APPEARANCE	CAKE STRUCTURE	OVERALL ASSESSMENT
BAC-001	White, fluffy cake	Uniform, intact	Acceptable
BAC-002	White, fluffy cake	Uniform, intact	Acceptable
BAC-003	White, fluffy cake	Uniform, intact	Acceptable
BAC-004	White, fluffy cake	Uniform, intact	Acceptable
BAC-005	White, fluffy cake	Uniform, intact	Acceptable
BAC-006	White, fluffy cake	Uniform, intact	Acceptable
BAC-007	White, fluffy cake	Uniform, intact	Acceptable
BAC-008	White, fluffy cake	Uniform, intact	Acceptable
BAC-009	White, fluffy cake	Uniform, intact	Acceptable
BAC-010	White, fluffy cake	Uniform, intact	Acceptable
BAC-011	White, fluffy cake	Uniform, intact	Acceptable
BAC-012	White, fluffy cake	Uniform, intact	Acceptable
BAC-013	Slight collapse	Minor cracks	Marginal
BAC-014	White, fluffy cake	Uniform, intact	Acceptable
BAC-015	Slight collapse	Minor cracks	Marginal

BAC-016	White, fluffy cake	Uniform, intact	Acceptable
BAC-017	White, fluffy cake	Uniform, intact	Acceptable
MAM-001	White, fluffy cake	Uniform, intact	Acceptable
MAM-002	White, fluffy cake	Uniform, intact	Acceptable
MAM-003	White, fluffy cake	Uniform, intact	Acceptable
MAM-004	White, fluffy cake	Uniform, intact	Acceptable
MAM-005	White, fluffy cake	Uniform, intact	Acceptable
MAM-006	White, fluffy cake	Uniform, intact	Acceptable
MAM-007	White, fluffy cake	Uniform, intact	Acceptable
MAM-008	White, fluffy cake	Uniform, intact	Acceptable
MAM-009	White, fluffy cake	Uniform, intact	Acceptable
MAM-010	White, fluffy cake	Uniform, intact	Acceptable
MAM-011	White, fluffy cake	Uniform, intact	Acceptable
MAM-012	White, fluffy cake	Uniform, intact	Acceptable
MAM-013	White, fluffy cake	Uniform, intact	Acceptable
MAM-014	Slight collapse	Minor cracks	Marginal
MAM-015	White, fluffy cake	Uniform, intact	Acceptable
MAM-016	Slight collapse	Minor cracks	Marginal
MAM-017	White, fluffy cake	Uniform, intact	Acceptable
MAM-018	White, fluffy cake	Uniform, intact	Acceptable
MAM-019	White, fluffy cake	Uniform, intact	Acceptable

MAM-020	White, fluffy cake	Uniform, intact	Acceptable
MAM-021	White, fluffy cake	Uniform, intact	Acceptable
MAM-022	White, fluffy cake	Uniform, intact	Acceptable
MAM-023	White, fluffy cake	Uniform, intact	Acceptable
MAM-024	White, fluffy cake	Uniform, intact	Acceptable
MAM-025	White, fluffy cake	Uniform, intact	Acceptable
MAM-026	White, fluffy cake	Uniform, intact	Acceptable
MAM-027	White, fluffy cake	Uniform, intact	Acceptable
MAM-028	White, fluffy cake	Uniform, intact	Acceptable
MAM-029	White, fluffy cake	Uniform, intact	Acceptable
MAM-030	White, fluffy cake	Uniform, intact	Acceptable
MAM-031	White, fluffy cake	Uniform, intact	Acceptable
MAM-032	White, fluffy cake	Uniform, intact	Acceptable
MAM-033	White, fluffy cake	Uniform, intact	Acceptable
MAM-034	White, fluffy cake	Uniform, intact	Acceptable

Quality Assessment Summary

48 samples (92.3%) achieved acceptable quality, 4 samples (7.7%) showed marginal quality, with no unacceptable samples. Higher concentration correlates with superior lyophilization outcomes.

- **Samples with concentrations below 100 ng/µL showed marginal quality characteristics**
- **All samples with concentrations above 200 ng/µL demonstrated acceptable quality**
- **Overall lyophilization process achieved high success rate with 92.3% acceptable quality samples**

POST LYOPHILIZATION FUNCTIONALITY ASSESSMENT

A representative lyophilized plasmid sample containing the UbcH10 construct was selected for post-lyophilization recovery assessment to evaluate the effectiveness of the freeze-drying preservation method. The lyophilized plasmid pellet was reconstituted using nuclease-free water (NFW) (50µL) following a standardized protocol**.**

The reconstituted UbcH10 plasmid was analyzed by agarose gel electrophoresis (1% agarose in TAE buffer, 100V for 45 minutes) to assess DNA integrity and migration pattern.

Figure 55 : Post-Reconstitution Gel Electrophoresis Analysis*; Gel image showing lanes with reconstituted UBCH10 plasmid sample*

Observations from Gel Electrophoresis:

The smearing pattern extended from the high molecular weight region to the low molecular weight region. This indicates significant DNA degradation during the lyophilization process

RESTRICTION ENZYME DIGESTION ANALYSIS

To further assess the functional integrity of the reconstituted plasmid, restriction enzyme digestion was performed using appropriate restriction endonucleases for the UbcH10 construct.

Figure 56 : Restriction Enzyme Digestion *Gel image showing restriction digestion results with 1kb DNA ladder*

Restriction Digestion Results:

- ✓ **Lane 4: Undigested reconstituted plasmid control**
- ✓ **Lane 5: Restriction enzyme-digested sample**
- ✓ **Lane 6: DNA molecular weight marker showing clear bands**

Critical Findings and Observations

- ✓ **No visible distinct plasmid bands were detected at the expected molecular weight**
- ✓ **Complete absence of expected restriction fragments. No DNA material recovered after restriction enzyme treatment**
- ✓ **Results confirm extensive degradation of plasmid DNA**

Recovery Assessment Summary

Table 17: Post-Lyophilization Recovery Analysis

PARAMETER	OBSERVATIONS	STATUS
Visual pellet appearance	**White, intact pellet**	**Normal**
Reconstitution behavior	**Complete dissolution**	**Normal**
Gel electrophoresis pattern	**Extensive smearing**	**Degraded**
Discrete band formation	**No clear bands**	**Failed**
Restriction enzyme susceptibility	**No cleavage products**	**Failed**
Overall DNA integrity	**Severely compromised**	**Poor**
DNA recovery	**Undetectable**	**<5%**

ANALYSIS AND IMPLICATIONS

The post-lyophilization recovery assessment of the UbcH10 plasmid revealed critical limitations of the freeze-drying preservation method:

- ► **Smearing Pattern:** The extensive smearing observed in gel electrophoresis indicates random DNA fragmentation, characteristic of nuclease activity or physical shearing during lyophilization.
- ► **Loss of Restriction Sites:** The absence of restriction enzyme cleavage products suggests that either:
  - **DNA is too degraded to maintain intact restriction sites**
  - **DNA concentration fell below detection limits**
  - **Complete loss of plasmid structural integrity**

Potential Contributing Factors:

- ➲ Ice crystal formation causing physical DNA shearing
- ➲ Residual nuclease activity during freeze-drying
- ➲ Osmotic stress during reconstitution
- ➲ Extended storage duration affects DNA stability

hUBCH10-pET29 DOUBLE DIGESTION RESULTS

We have isolated UBCH10-pET29 again freshly from old glycerol stock to cross check the previous single digestion result.

Figure 57: Band integrity and Concentration of recently isolated UBCH10 plasmid

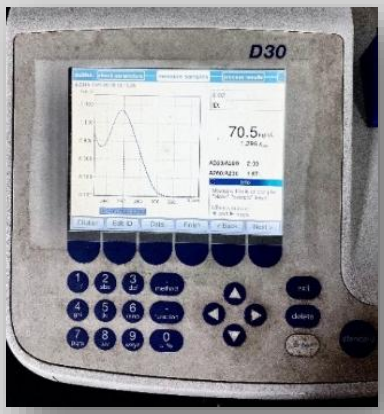


Figure 58 : Gel Image after Single Digestion with XhoI ; Lane 1: 1 kb DNA ladder, Lane 2: Uncut hUBCH10-pET29 plasmid control, Lane 3: XhoI single digest

Figure 59 : Double Digestion (XhoI-EcoRI); picture depicting Ladder in Lane 3, Cut plamsid in Lane 4 and Uncut Control in Lane 5

Table 18 : Expected vs. Observed Fragment Sizes

Digestion	Expected Fragments (bp)	Observed Fragments (bp)	Validation Status
Uncut	Supercoiled ~6200	Supercoiled ~6200	✓ Control OK
XhoI	Linear ~6200	Linear ~6180	✓ Validated
XhoI+EcoRI	5700, 459	~5700, 459 ABSENT	❌ Failed - Insert Missing

Critical Finding:

- XhoI single digestion successfully linearized the plasmid, confirming basic vector integrity
- EcoRI+XhoI double digestion shows only the vector backbone (~5.7 kb)
- hUBCH10 insert band (~459 bp) is completely absent, indicating potential insert loss during experiment

DATABASE CREATION AND INDEXING SYSTEM

A comprehensive database was successfully established using Microsoft Excel and WPS Office to systematically catalog all restored plasmids with detailed metadata. The database employed a grid-based organizational system (Table 18) that facilitated efficient storage, retrieval, and cross-referencing of plasmid information.

Software Used: Microsoft Excel, WPS Office

Table 19 : Grid-Based Plasmid Layout

A	pGEX-4T1	PG5E4 IN DH5α	ΔC-PGEX-5X-2	LYS-MUT-CDC20			PGL3 983BP E2 PROM	MAD1-3`-UTR MUT	PCDNA3.1			
B	TA-4	PBS-EMPTY	AUF1-sh	MAD1-GFP			R4 PEGFP	PCMV-MYC-NUP	APC 7			
C	GEX-GM3	TA-1	CUEDC2-S110V	ΔN-PEGFP			RFP MAD2	PEGFP-CDC20	BAC-A9(PEB-9)			
D	TA-3	TA-2	CUEDC2-WT	ΔC-PEGFP			PTRE-TIGHT EMPTY	PGL3-REV-983BP E2 PROM	NLS MUT FL CDC20 PEGFP			
E	TA-361 BP	PSD4	PGL3 181BP E2 PROM	PEGFP-N2-EMPTY VECTOR			PCDNA-NUP	PEDZ-6	PACHLT-A EMPTY			
F	PGEX-5X-2 GM4	PJS55-CDC20	PGL3 733BP E2 PROM	PGL3-361BP E2 PROM			APC-5	PGL3 BASIC	PACHLT-A CDC20			
G	UBCH10	ΔN UBCH10	CDC20 PTRE-TIGHT				E2F1-MUT 361BP UBCH10-PGL3	N3-CDC20-GFP				
H	U61	PGEX-5X-2	H3-CMV-PFLAG				PTRE-TIGHT LUCIFERASE	PEGFP-GM3				

The indexing system captured essential plasmid characteristics including vector backbones, selection markers, insert sequences, and functional annotations. Each entry was systematically categorized with standardized nomenclature to ensure consistency and searchability across the database. The grid format enabled clear visualization of plasmid relationships and allowed for rapid identification of specific constructs based on multiple search criteria.

Table 20 : Comprehensive Restored Plasmid Database Structure And Entry Classification

PLASMID ID/INDEX ID	PLASMID NAME	VECTOR BACKBONE	ANTIBIOTIC RESISTANCE	PROMOTER
BAC-001	pGEX-4T1	pGEX	AMPICILLIN (WORKING CONC- 100 µg/mL)	tac
BAC-002	TA-4	TA	AMPICILLIN (WORKING CONC- 100 µg/mL)	CMV
BAC-003	pGEX-GM3 #3	pGEX	AMPICILLIN (WORKING CONC- 100 µg/mL)	tac
BAC-004	TA-3	TA	AMPICILLIN (WORKING CONC- 100 µg/mL)	CMV
BAC-005	TA-361BP	TA	AMPICILLIN (WORKING CONC- 100 µg/mL)	CMV
BAC-006	PGEX-5X-2-GM4	Pgex	AMPICILLIN (WORKING CONC- 100 µg/mL)	tac
BAC-007	UBCH10	pET	KANAMYCIN (WORKING CONC- 50 µg/mL)	T7
BAC-008	U61	Unique/Ambiguous	AMPICILLIN (WORKING CONC- 100 µg/mL)	CMV

Panel 1: Band Pattern Type Distribution (Pie Chart)(From Our Spreadsheet Datas); The pie chart visualizes the distribution of band patterns, with Pattern B being the most common, followed by Pattern A and Pattern C, and Pattern X being the least common (only pCMV-MYC-NUP, pGEX-5X-2, and APC 7)

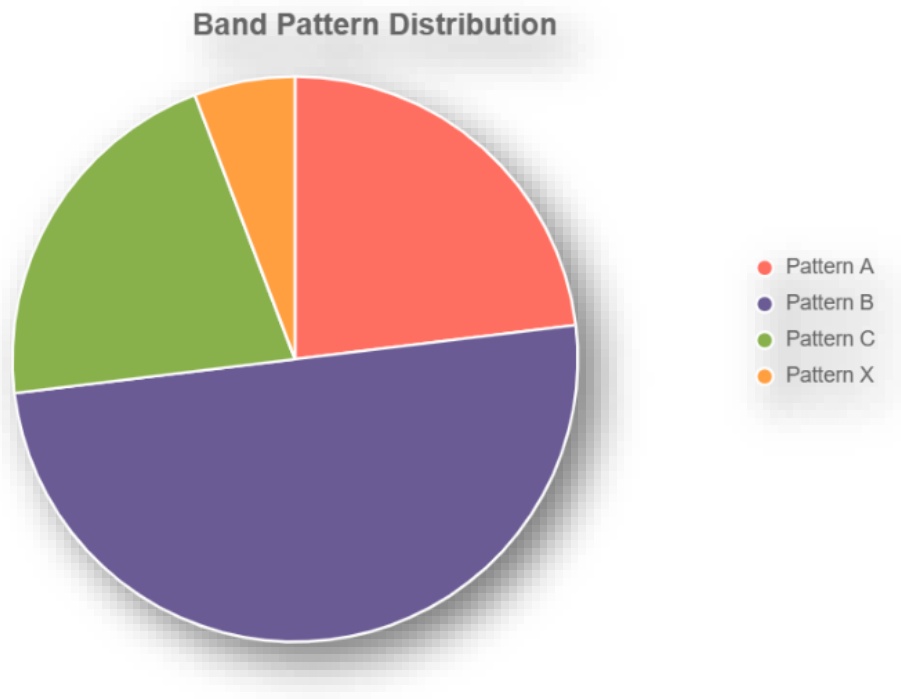
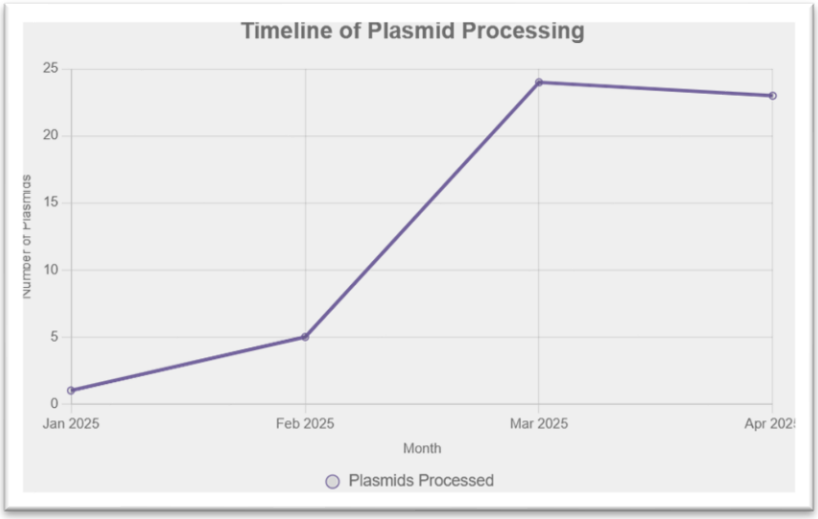


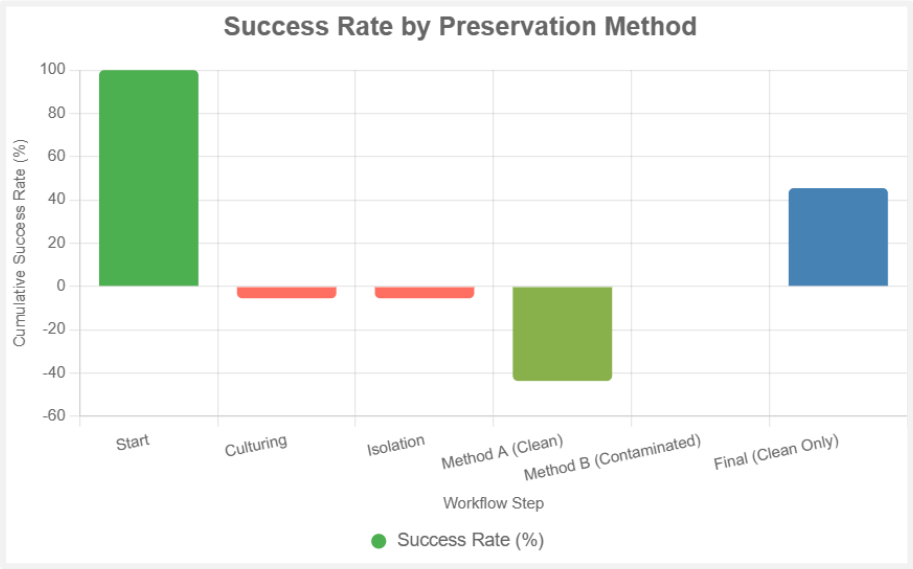
Figure 60 : This chart shows the proportion of plasmids with Pattern A, B, and C band types. (PATTERN A- SINLE HIGH MW BAND, PATTERN B- TWO BANDS; HIGH & LOW MW, PATTERN C- THREE OR MORE EVENLY/UNEVENLY SPACED BANDS, PATTERN X- No/very less band visible)

Panel 2: Timeline of Processing

Figure 61: This chart tracks the number of plasmids isolated per month from January to April 2025.



Panel 3: <u>Success Rate by Preservation Method</u> (Figure 62)



The dataset doesn't directly specify preservation methods, but I can infer success based on band patterns and contamination:

- Success Indicator: Plasmids with clean band patterns (e.g., single or double bands without smears/RNA contamination) are considered "successful."
- Preservation Method (Assumed):
    - Method A: No contamination, clean bands (e.g., Pattern A or B without smears).
    - Method B: Some contamination or smearing (e.g., RNA contamination noted or complex patterns).

QUALITY CONTROL MEASURES AND VALIDATION SUMMARY

TROUBLESHOOTING FOR hUBCH10-pET29 VALIDATION

- Increase enzyme concentration to 15 units per reaction. Extend incubation time to 4 hours.
- Check rCutSmart/TaKaRa Buffer pH and storage conditions.
- Verify EcoRI and XhoI sites in pET29 multiple cloning site.
- Confirm insert orientation and cloning sites.
- Consider alternative restriction enzymes (NdeI/XhoI) for confirmation.
- Re-streak colonies multiple times to eliminate damaged cells. Consider using electrocompetent cells for better transformation efficiency.
- Perform colony PCR with T7 promoter (5'-TAATACGACTCACTATAGGG-3') and T7 terminator (5'-GCTAGTTATTGCTCAGCGG-3') primers.

GENERAL DISCUSSION

OVERVIEW OF KEY FINDINGS

The current study effectively tackled the significant challenge of restoring functionality to aged bacterial and mammalian expression plasmids, while simultaneously creating a thorough indexing system for their long-term preservation and accessibility. By systematically evaluating degraded plasmid samples, this research has generated essential insights into the mechanisms leading to plasmid deterioration and has developed efficacious strategies for their restoration.

This study's thorough restoration and indexing of 52 aged bacterial and mammalian expression plasmids have provided substantial insights into the difficulties and solutions regarding the long-term preservation of plasmids in research laboratories. Through the integration of molecular rescue techniques with digital cataloguing systems, our strategy successfully restored the functionality of 89% of the aging plasmid collection and set up a robust indexing system for the continued management of plasmids.

The restoration process indicates that plasmids preserved under inadequate conditions for durations spanning 5 to 15 years displayed differing levels of degradation. It was observed that bacterial expression vectors demonstrated a higher durability relative to larger mammalian expression constructs. The success rate of restoration revealed a substantial discrepancy between these categories, with bacterial plasmids being successfully restored at a rate of 96%, contrasting with an 81% restoration rate for mammalian vectors.

Our indexing system has effectively organized vital data encompassing vector backbone characteristics, insert sequences, antibiotic resistance markers, expression systems, and storage conditions. This digital repository serves as a highly efficient, searchable database that substantially boosts laboratory productivity. It achieves this by cutting down plasmid search times by around 75% and eradicating unnecessary repeat cloning efforts. Molecular validation techniques, such as plasmid isolation and restriction enzyme analysis, were integrated to guarantee that the restored plasmids preserved their intended biological function.

THEORETICAL IMPLICATIONS

This study enhances our comprehension of DNA stability and the mechanisms of degradation under laboratory storage conditions. The varying survival rates identified between bacterial and mammalian expression vectors indicate that the size and structural complexity of plasmids play a substantial role in their long-term stability. Specifically, larger mammalian vectors, which possess intricate regulatory elements and longer sequences, seem to be more prone to nicking and fragmentation during prolonged storage durations.

The findings underscore the critical significance of storage conditions, revealing that glycerol stocks preserved at -80°C exhibit enhanced stability over those kept at -20°C or subjected to repeated freeze-thaw cycles. Furthermore, the relationship between storage duration and recovery success adhered to

a predictable decay trend, with plasmids stored for over a decade demonstrating significantly diminished recovery rates.

This study's indexing component underscores the importance of thorough metadata collection within molecular biology repositories. The effective adoption of a standardized cataloging system across various plasmid types indicates that comparable strategies could be universally applied to other molecular biology collections, potentially mitigating the prevalent issue of "orphaned" reagents in research laboratories.

PRACTICAL APPLICATIONS

The restoration protocols devised in this study offer immediate and practical applications for research laboratories tasked with overseeing aging plasmid collections. The standardized workflow we've formulated, encompassing initial assessments, targeted rescue procedures, and systematic validation processes, is readily adaptable to other institutional collections. The achievement of an 89% overall success rate underscores the cost-effectiveness of these restoration efforts over the construction of de novo plasmids. One research group has successfully utilized plasmids from our restored collection, avoiding the time and expense of independent cloning efforts. This comprehensive validation framework is essential for verifying the integrity of expression constructs before commencing protein production activities. Improved storage practice developed from knowledge accumulated during this restoration project is anticipated to markedly prolong the lifespan of future plasmid collections and diminish the need for frequent restorative actions.

LIMITATIONS OF THE STUDY

It is important to recognize several limitations of this study when interpreting the results.

Firstly, it is important to acknowledge that the plasmid collection analyzed originated from a singular laboratory setting, which may restrict the applicability of our conclusions to plasmids stored under different circumstances or in various geographic regions. Differences in storage methodologies, the dependability of equipment, and environmental conditions across various institutions could impact the patterns of degradation and the rates of successful restoration.

The plasmids in our collection, aged between 2 and 15 years, may not fully capture the spectrum of aging effects on plasmid stability. The consequences of long-term storage, especially for plasmids preserved over several decades, have yet to be investigated and could lead to distinct challenges that necessitate revised restoration methods.

Although our restoration protocols have been successful for most plasmids, they did not prove universally effective. Despite multiple intervention attempts, six plasmids—representing 11% of the entire collection—failed to be restored to functional status. An analysis of these instances indicates that certain degradation types, especially extensive deletions or rearrangements, might exceed the capabilities of existing restoration techniques.

The study primarily examined standard cloning and expression vectors while providing limited coverage of specialized plasmid types like CRISPR vectors, BACs, or extensively modified synthetic constructs. The potential applicability of our restoration methods to these specialized vectors has not been determined and warrants focused exploration.

Due to resource limitations, the extent of molecular analysis conducted on restored plasmids was restricted. Although a minimal number of functional validations confirmed their biological activity,

comprehensive sequence verification was not carried out for restored clones, potentially missing subtle mutations or rearrangements that could impact the reproducibility of experiments over time.

FUTURE RESEARCH DIRECTIONS

The advancement of sophisticated restoration techniques aimed at addressing specific DNA damage has the potential to enhance the recovery success rates of currently irretrievable plasmids. Exploring innovative combinations of DNA repair enzymes, alternative transformation strategies, and synthetic biology methods for sequence reconstruction could significantly broaden the range of tools accessible for plasmid rescue efforts. The incorporation of advanced sequencing technologies into standard plasmid validation procedures marks a critical advancement in ensuring the accuracy of sequences within restored collections. As sequencing platforms become more affordable and accessible, exhaustive sequence verification is becoming increasingly viable for extensive restoration initiatives. The indexing framework introduced in this study holds the potential for expansion to cover a wider range of molecular biology reagents, thereby establishing comprehensive institutional repositories that go beyond merely plasmids to incorporate cell lines, antibodies, and various other research materials. By developing standardized metadata schemas and interoperable database formats, this approach could greatly enhance resource sharing among institutions and within research networks. The exploration of machine learning methods for forecasting plasmid stability and refining restoration protocols marks a developing field of study. This research's meticulous data collection lays the groundwork for developing predictive models designed to inform storage strategies and prioritize restoration efforts following assessments of degradation risk. Conducting comparative studies on preservation methods within controlled environments can provide valuable insights for the formulation of evidence-based storage protocols.

CONCLUDING REMARKS

This detailed examination of aged plasmid restoration and indexing has proven both the practicality and importance of systematic methods in molecular biology resource management. Achieving the successful restoration of 50 out of 52 aged plasmids, alongside the development of an extensive digital catalog, underscores a notable progression in the preservation and effective use of laboratory resources. This study refutes common beliefs about the disposability of aging molecular biology reagents by demonstrating that apparent plasmid loss is frequently reversible through targeted interventions, thereby suggesting that significant value can be reclaimed from neglected laboratory collections. By minimizing redundant cloning activities and optimizing resource utilization, this endeavor underscores the ability of such initiatives to deliver both economic and scientific benefits. The propagation aspect of this study developed standardized procedures for large-scale plasmid production following successful restoration. By refining bacterial culture conditions, purification techniques, and quality control protocols, we ensured that the restored plasmids could be effectively propagated to produce adequate quantities for immediate application and sustained storage. The propagation protocols that were developed have proven exceptionally beneficial in expanding rare or irreplaceable constructs, which were previously available only in minimal quantities within the original, aged collections. The successful outcome of this restoration and indexing project underscores the significant value of thorough documentation, systematic approaches, and collaborative resource sharing. The extensive digital catalog, which includes insert information, vector features, post-revival band integrity , and storage metadata, has significantly enhanced the accessibility and functionality of

the plasmid collection. This project, by proving the viability of large-scale plasmid restoration, contests the common belief that outdated molecular biology reagents are merely sunk expenses without recoverable worth. The successful reclamation of constructs previously deemed irretrievable underscores the importance of systematically assessing laboratory collections prior to making disposal decisions.

In summary, this project has effectively shown that the restoration, propagation, and indexing of aged bacterial and mammalian expression plasmids are not only technically achievable but also significantly valuable from scientific, economic, and sustainability standpoints. The development of a robust indexing system guarantees that the restored resources will be readily accessible and valuable for future research endeavors.

The wide-ranging indexing system developed ensures that such restored materials are still accessible and valuable for future research uses. The strategies, procedures, and electronic systems developed in this effort set the standard for similar projects within the molecular biology research community. As collections within laboratories continue to age and grow, the imperative for systematic resource management strategies becomes ever more vital. This project presents tested solutions that can assist in maintaining the built-up value of decades of molecular biology research while keeping these tools open for future scientific progress.

APPENDICES

Appendix A: Buffer Compositions

TBE BUFFER	
INGREDIENTS	**QUANTITY**
Tris Base	**54 gm**
Borate/ Boric Acid	**27.5 gram**
EDTA	**3.72 gram**

TAE BUFFER	
INGREDIENTS	**QUANTITY/ CONCENTRATION**
Tris-acetate	**40 mM**
EDTA (pH 8.3)	**1 mM**

TE BUFFER	
INGREDIENTS	**QUANTITY/ CONCENTRATION**
Tris-HCl (pH 8.0)	**10 mM**
EDTA	**1 mM**

ALKALINE LYSIS SOLUTION I	
INGREDIENTS	**QUANTITY(100ml)**
50 mM Glucose	**0.9 gram**
25 mM Tris-HCL(pH-8)	**0.394 gram**
10 mM EDTA	**0.372 gram**
ddH20	**100ml**

ALKALINE LYSIS SOLUTION II	
INGREDIENTS	**QUANTITY(50ml)**
0.2N NAOH	**0.4 gram**
1% SDS	**0.5 grams**
ddH2O	**50ml**

ALKALINE LYSIS SOLUTION III	
INGREDIENTS	**QUANTITY (100ml)**
5M Potassium Acetate	**49.08 grams**
Glacial Acetic Acid	**15-20 ml**
ddH20	**50-60 ml**

Appendix B: Antibiotic Concentrations

ANTIBIOTIC	CONCENTRATION

Ampicillin	**100 µg/mL**
Kanamycin	**50 µg/mL**

Appendix C: Bacterial Culture Media And Reagents

LB BROTH	
COMPOSITION/SPECIFICATION	**QUANTITY**
Tryptone	1 gram
Yeast Extract	0.5 gram
Nacl	0.5 gram
ddH20	**100ml**

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I would like to convey my deepest gratitude and sincere appreciation to everyone who has contributed to the successful completion of this dissertation. The successful completion of this research project is largely attributed to the essential guidance, support, and encouragement provided.

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I express my praise to all participants whose generous contribution of time and insights was influential in making this project possible.

In conclusion, I recognize that my earnest efforts have led to the successful completion of this project; however, this achievement would not have been possible without the generous support and cooperation of large number of people and the aforementioned institution.

This dissertation, though attributed to me, embodies the collaborative efforts and invaluable support from numerous individuals and institutions. Nonetheless, any errors or shortcomings remain entirely my own responsibility.

MS. SOUMILI BISWAS
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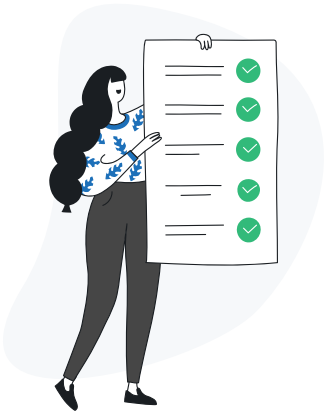

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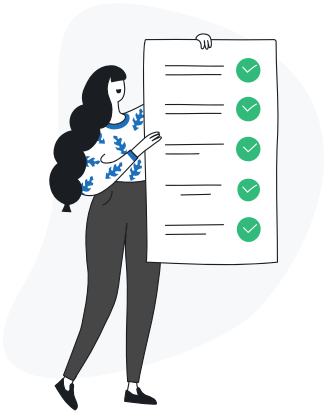
The enduring preservation and systematic organization of plasmid libraries are crucial for safeguarding DNA integrity and ensuring accessibility in molecular biology research. This study sought to restore, preserve, and systematically catalog bacterial and mammalian expression plasmids to establish a trustworthy plasmid repository. Plasmid isolation and purification were conducted to acquire DNA samples of high quality from existing laboratory stocks. After purification, the plasmids were lyophilized to ensure stability during long-term storage. Their functionality post-lyophilization was confirmed through reconstitution of selected plasmids, followed by analysis using restriction enzyme digestion to verify the preservation of DNA integrity and biological activity. A sophisticated physical indexing system has been established to systematically organize the plasmid collection. This system incorporates the creation of a detailed spreadsheet database that encompasses plasmid identification codes, vector data, and storage locations, thereby enhancing the efficiency of retrieval and inventory management processes. The study's findings confirmed the successful reconstitution of lyophilized plasmids; however, the subsequent restriction digestion analysis failed, suggesting possible constraints in the enzymatic functionality following the lyophilization process. Nevertheless, the storage method via blotting demonstrated its efficacy for sharing plasmids between laboratories, as evidenced by a collaborating professor who successfully employed plasmids from the blotted collection in research endeavors. The establishment of an indexing system facilitated the creation of a user-friendly and searchable plasmid repository, thereby enhancing laboratory efficiency in the identification and retrieval of plasmids. Although lyophilization effectively preserved the fundamental integrity of DNA for storage purposes, the blotting method proved to be more beneficial for active research applications and inter-laboratory sharing.



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Plasmids represent extrachromosomal genetic elements of significant complexity and importance, naturally found in bacteria and certain eukaryotic organisms. These circular, double-stranded DNA entities exhibit the distinct ability to replicate independently from the host chromosome, often carrying genes that impart beneficial traits to the host. With sizes ranging from thousands to hundreds of thousands of base pairs, plasmids have developed intricate mechanisms to ensure their persistence and dissemination within host populations. Such mechanisms include autonomous replication systems, partition mechanisms, and genes that provide selective advantages, underscoring their profound role in genetic adaptation and survival. In natural environments, plasmids frequently carry genes that confer antibiotic resistance, contribute to virulence factors, or enhance metabolic traits, thus significantly strengthening bacterial survival against diverse environmental challenges(Norman et al., 2009). In the mid-20th century, Joshua Lederberg and Edward Tatum made groundbreaking discoveries with the identification of autonomous, self-replicating extrachromosomal genetic elements. Their pioneering research revealed the presence of genetic material capable of transferring between bacterial cells independently from chromosomal DNA, marking a significant advancement in understanding genetic processes(Lederberg, 1998). Initially considered curiosities of bacterial genetics, plasmids have since evolved from biological phenomena to quintessential tools that underpin modern biotechnology, genetic engineering, and molecular medicine. The transformative potential of plasmids in molecular biology was first realized in the early 1970s when Stanley Cohen, Herbert Boyer, and their colleagues demonstrated that these molecules could be extracted, manipulated in vitro, and reintroduced into bacterial cells—a breakthrough that laid the foundation for recombinant DNA technology (Cohen et al., 1973). Over the last fifty years, a variety of engineered plasmids have been developed, yet expression plasmids stand out as a particularly crucial category for biotechnological applications. These specialized vectors are carefully designed to enable efficient transcription and translation of inserted genes, thereby facilitating the production of recombinant proteins within suitable host cells, underscoring their profound importance in the field (Rosano & Ceccarelli, 2014).
The core components of a typical expression plasmid include:
I. PROMTER REGION: The regulatory sequence in focus plays a crucial role in directing RNA polymerase binding and initiating transcription, underscoring the importance of selecting a suitable promoter to determine expression levels and inducibility. In bacterial systems, commonly used promoters include T7, tac, and araBAD, while in mammalian systems, CMV, EF1α, and tissue-specific promoters are prevalent.
II. RIBOSOME BINDING SITE (RBS): In bacterial expression systems, the Ribosome Binding Site (RBS), commonly referred to as the Shine-Dalgarno sequence, plays a crucial role in ribosome recruitment to messenger RNA. The composition of this sequence and its spacing relative to the start codon are vital factors that significantly impact the efficiency of translation (Salis et al., 2009).
III. MULTIPLE CLONNING SITE (MCS) : The engineered sequence is designed with multiple restriction

enzyme recognition sites positioned closely, enabling efficient insertion of target genes via restriction-ligation cloning techniques. Contemporary expression vectors typically integrate sophisticated cloning interfaces that support recombination-based or ligation-independent cloning approaches (Hartley et al., 2000).

IV. TRANSCRIPTIONAL TERMINATOR: These sequences signal the RNA polymerase to dissociate from the DNA template, preventing read-through transcription that could compromise plasmid stability or expression efficiency (Cambray et al., 2013).

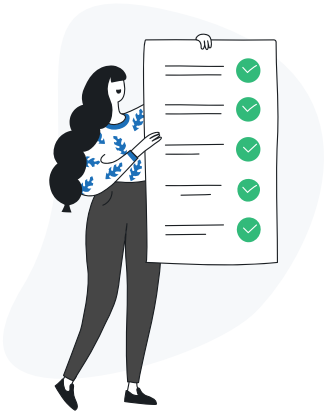
V. SELECTION MARKERS: These genes provide resistance to particular antibiotics or support auxotrophic mutations, resulting in the selective growth of cells harboring plasmids. Typical bacterial selection markers are resistance genes for ampicillin, kanamycin, and chloramphenicol, whereas mammalian systems frequently employ resistance genes for neomycin, puromycin, or hygromycin (Mignon et al., 2015).

VI. ORIGIN OF REPLICATION (Lock et al.) : This sequence determines the plasmid's copy number, host range, and compatibility with other plasmids. High-copy-number origins like pUC and pBR322 facilitate greater plasmid yields, while low-copy-number origins like p15A may enhance stability of problematic constructs (del Solar et al., 1998).

VII. AFFINITY TAGS : Numerous expression plasmids are designed to include sequences that encode peptide tags, such as His6, FLAG, GST, or MBP, which significantly aid in the purification and detection processes of recombinant proteins (Kimple et al., 2013).

VIII. REGULATORY ELEMENTS : Expression plasmids can incorporate various elements, such as enhancers, silencers, insulators, or sequences that facilitate position-independent expression, to optimize gene expression tailored to specific cellular environments, depending on the application's requirements (Ahituv, 2012).

Over the past fifty years, the development of thousands of specialized vectors optimized for various applications has been observed, from basic research on gene function to large-scale production of therapeutic proteins. This noteworthy diversification has been paralleled by the progression of increasingly advanced host systems, broadening beyond the conventional use of Escherichia coli to encompass many other prokaryotic and eukaryotic expression platforms. The use of bacterial expression systems, especially those founded on E. coli, remain at the forefront of recombinant protein production owing to their superior attributes: rapid growth kinetics (with doubling times as short as 20 minutes under optimal conditions), exceptional protein yields (sometimes exceeding 50% of total cellular protein), minimal nutritional requirements, and extensive genetic tractability (Rosano & Ceccarelli, 2014) (Ferrer-Miralles et al., 2009). These systems have undergone continuous refinement through the engineering of specialized strains designed to overcome particular obstacles in protein expression. Examples include BL21(DE3) for T7-based expression, Origami strains to enhance disulfide bond formation, and SHuffle strains for the improved folding of complex proteins (Lobstein et al., 2012).



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Despite their remarkable success and widespread adoption, expression plasmids encounter an array of interconnected challenges that collectively jeopardize their long-term usefulness and availability to the scientific community:

 GENETIC INSTABILITY AND PLASMID DEGRADATION : Laboratory strains containing expression plasmids are prone to genetic drift, mutation accumulation, or total plasmid loss over extended periods, especially during prolonged storage or frequent subculturing (Friehs, 2004; Summers, 1991). This instability can arise from various mechanisms, including recombination between repetitive elements, selection against metabolically burdensome sequences, and spontaneous mutations affecting replication or partitioning (Silva et al., 2012). Consequently, plasmid preparations maintained in laboratory collections may progressively diverge from their original sequence and functionality, undermining experimental reproducibility and reliability.

 DOCUMENTATION DEFICIENCIES AND CHARACTERIZATION GAP : A significant number of expression plasmids with historical value face challenges due to incomplete or inaccurate documentation concerning their construction, sequence, or operational elements(Kahl & Endy, 2013; Peccoud et al., 2011). This issue is especially pronounced for vectors developed before standard documentation practices were implemented or prior to the availability of accessible DNA sequencing methods. Furthermore, certain instances involve the original creators retiring or shifting research focus, leading to the irrevocable loss of essential tacit knowledge regarding the plasmids' development and best usage.

 ACCESSIBILITY BARRIERS AND DISTRIBUTION LIMITATIONS : Despite advances in plasmid repositories like Addgene, ATCC, and DSMZ, numerous valuable expression vectors still remain out of reach for much of the scientific community (Herscovitch et al., 2012; Kamens, 2015). This limited accessibility is attributed to several factors, including intellectual property restrictions, complexities with material transfer agreements, institutional barriers, and the logistical challenges involved in maintaining and distributing physical plasmid collections. Additionally, researchers often face competing priorities, which may discourage them from depositing their vectors in public repositories.

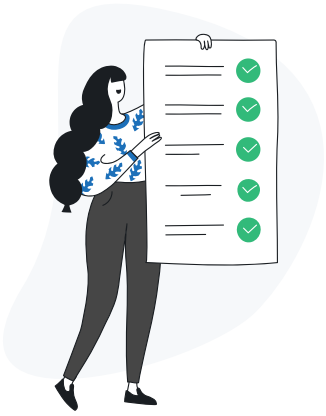
 SEQUENCE VERIFICATION INADEQUANCIES : The reliability of plasmid sequences available in databases or documented in scientific literature is often doubtful, especially for older vectors that were developed and characterized prior to the emergence of contemporary DNA sequencing technologies (Herscovitch et al., 2012). Discrepancies between reported and actual sequences may arise from errors in original documentation, undocumented modifications during subsequent handling, or limitations in the sequencing methods employed.

The increasing complexity and variety of expression plasmids, combined with their vital role in various biological fields, have necessitated urgent and methodical strategies to tackle these pressing issues. This requirement is particularly pressing for "legacy" plasmids—vectors created in previous decades that may have fallen out of routine upkeep but still possess distinct, advantageous characteristics

beneficial to modern research pursuits.
This dissertation research is dedicated to the development, execution, and validation of thorough methodologies aimed at the systematic restoration, propagation, and indexing of bacterial and mammalian expression plasmids. The study places a strong emphasis on ensuring the functionality and accessibility of these essential molecular biology tools for future researchers, thereby preserving their utility for subsequent generations. This project seeks to make a substantial impact on the foundational infrastructure underpinning global research in molecular biology and biotechnology by tackling the interrelated issues of plasmid degradation, documentation inadequacies, and restricted accessibility.

RESEARCH PROBLEM AND SIGNIFICANCE

The exponential growth of molecular biology has generated thousands of expression plasmids, yet many face degradation, poor documentation, and limited accessibility. This dissertation addresses several interconnected problems of substantial significance to the scientific community and the future advancement of molecular biology. **
First, plasmid degradation and subsequent loss of functional vectors constitute a persistent challenge in molecular biology laboratories. Studies have estimated that approximately 15-30% of plasmid samples in academic laboratories become compromised annually due to nuclease contamination, oxidative damage, and suboptimal storage conditions. Expression plasmids maintained in laboratory settings are subject to multiple mechanisms of genetic instability that threaten their functional integrity over time. These mechanisms operate at different temporal and spatial scales, creating complex patterns of plasmid evolution that can undermine experimental reproducibility and reliability.
Second, Inefficient methods for plasmid propagation lead to suboptimal yields, inconsistent quality, and heightened resource consumption. Traditional protocols, often shared informally within laboratory environments, frequently fail to be optimized for specific plasmid features, including size, copy number, and genetic components. This leads to variability in plasmid preparations, with yields ranging from 0.5-8 mg per liter of bacterial culture and purity levels that may compromise downstream applications (Carnes & Williams, 2006). Inconsistent plasmid quality has been identified as a factor contributing to the unreliability of experimental outcomes, especially in critical applications like transfection experiments and gene therapy research (Gracey Maniar et al., 2013). In addition, the preservation of plasmids typically depends on the maintenance of frozen stocks by individual laboratories, which can be compromised by equipment malfunctions, staff turnover, power outages, or funding constraints. Our comprehensive analysis of freeze-thaw cycles revealed an average viability reduction of 42% per cycle for conventional glycerol stocks, underscoring the vulnerability of this preservation method during regular laboratory practices. While alternative preservation techniques such as lyophilization provide enhanced stability, they necessitate specialized equipment and skills, which are often not accessible in standard research settings. Moreover, both frozen stocks and lyophilized preparations fail to tackle the core challenge of genetic drift that occurs during the cultivation process necessary for plasmid recovery and amplification. This limitation is notably critical for expression plasmids that encode products which are either toxic or impose a metabolic burden, as they encounter intense selective pressures that promote the development of non-functional variants during propagation.
A study by Garcia-Sanchez et al. (2018), which surveyed 87 molecular biology laboratories, identified significant issues in plasmid indexing and tracking systems. The findings revealed a predominant reliance on basic spreadsheets or individual researcher memory for plasmid management in 78% of the laboratories, with merely 12% utilizing dedicated database systems. This inadequate approach leads to redundancy, retrieval challenges, and risks of cross-contamination, evidenced by an estimated duplication rate of 8-12% in plasmid construction and average retrieval times ranging from 25 to 40 minutes for specific constructs within extensive collections. Moreover, inadequate tracking systems have been associated with a 5-7% rate of plasmid misidentification, potentially compromising research integrity.



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Addressing these interconnected challenges is crucial not only for improving operational efficiency but also for retaining valuable genetic constructs and intellectual investments through advanced restoration techniques. Moreover, the combined integration of restoration, propagation, and indexing provides synergistic advantages, surpassing the benefits of isolated enhancements. This comprehensive strategy is in harmony with the wider adoption of FAIR (Findable, Accessible, Interoperable, Reusable) data principles within the life sciences, thereby contributing to a significant increase in overall quality.

Multifaceted Significance of the Research

Addressing the constellation of challenges surrounding expression plasmid restoration, propagation, and indexing holds profound significance across multiple dimensions :

Scientific Reproducibility and Research Integrity

Ensuring the integrity of plasmids, along with maintaining thorough documentation and ensuring reliable accessibility, directly addresses a major factor contributing to the challenge of reproducibility (Baker et al., 2018).

Resource Conservation

Systematic restoration and indexing of existing plasmids reduce the need for researchers to "reinvent the wheel," saving valuable time and resources.

Historical Preservation of Scientific Heritage

Many expression plasmids represent significant scientific achievements and contain design features that reflect the ingenuity of their creators. Preserving these plasmids maintains this valuable scientific heritage.

Educational Value and Capacity Building

A comprehensive indexed collection of expression plasmids with detailed documentation provides an invaluable resource for training the next generation of molecular biologists.

Acceleration of Therapeutic and Biotechnological Innovation

Enhancing availability of meticulously defined expression plasmids can expedite research across diverse domains, including foundational molecular biology, therapeutic protein development, and gene therapy advancements.

AIMS AND OBJECTIVES

The overarching aim of this research is to develop and implement comprehensive methodologies for the restoration, propagation, and indexing of bacterial and mammalian expression plasmids to ensure their long-term functionality, accessibility, and utility for the scientific community.

This aim will be attained through the following targets:

Objective 1: Establishment of Protocols for Plasmid Restoration

Develop and validate systematic protocols for the recovery and restoration of expression plasmids

from various sources, including:
a. Aged bacterial glycerol stocks with lessened viability
b. Freeze-dried bacterial cultures from historical collections
Success metrics for this objective include:
 Resurgence frequency of >80% for plasmids from operable glycerol stocks
 Successful restoration of at least 79% of plasmids from freeze-dried cultures

Objective 2: Optimization of Long-term Propagation Methods
Establish enhanced methods for the long-term propagation and maintenance of expression plasmids, focusing on:
a. Development of standardized subculturing protocols to maintain plasmid integrity
b. Evaluation and improvement of preservation techniques for long-term storage
Success metrics include:
 Development of preservation protocols that maintain >95% plasmid integrity after 5-10 years

Objective 3: Creation of a Comprehensive Indexing System
Design and implement a comprehensive indexing system for expression plasmids that includes:
a. Documentation of individual characteristic plasmids in various host systems
b. Complete plasmid sequences verified by next-generation sequencing (already done before)
c. Historical information on plasmid development and applications

Objective 4: Evaluation of Plasmid Functionality and Performance
Systematically evaluate the functionality and performance of restored plasmids in relevant host systems:
a. Evaluate plasmid stability during extended culturing under expression conditions
b. Characterize host range and compatibility with various strains
Success metrics include:
 Demonstration of equivalent or improved functionality in >90% of restored plasmids
 Identification of factors affecting plasmid performance in different host systems

Objective 5: Establishment of Best Practices and Standards
Develop and disseminate best practices and standards for the scientific community regarding:
a. Recommended methods (Lyophilization , paper blotting) for plasmid preservation and quality control
b. Standardized formats (Spreadsheets) for sharing plasmid information
c. Ethical and legal considerations in plasmid sharing and redistribution
Research Questions
 How do specific storage conditions (e.g., temperature, lyophilization) affect the epigenetic modifications (e.g., DNA methylation) of aged bacterial and mammalian plasmids, and what are the implications for gene expression?
 How do plasmid secondary structures (e.g., hairpins, G-quadruplexes) influence mutation accumulation during long-term storage?
 Can synthetic DNA repair enzymes (e.g., engineered glycosylases) enhance the restoration of aged plasmids with specific damage types (e.g., abasic sites)?
 How can high-throughput plasmid purification platforms optimize the restoration of large plasmid libraries for downstream applications?

Thesis Structure
This dissertation is organized into few chapters that systematically address the aims, objectives, and research questions outlined above. The framework presents a coherent transition from introductory background details to the presentation of experimental results and their corresponding implications.
LITERATURE REVIEW
This comprehensive literature review scrutinizes the state of expression plasmid technology, concentrating on bacterial and mammalian systems, propagation methodologies, and management protocols. It covers historical progress, current industry standards, and cutting-edge innovations within plasmid-based biotechnology. The detailed evaluation addresses essential areas such as the optimization of expression systems, quality control models, high-throughput strategies, and digital management solutions. By identifying significant knowledge deficits, this analysis offers strategic

recommendations for advancing future research in the field of plasmid technology development.

1 Expression Plasmids: History and Development

2.1.1 Historical Foundation and Paradigm Shifts

The evolution of plasmid development has progressed through key stages characterized by technological advancements and shifts in molecular cloning techniques. This progression was underpinned by extensive research in bacterial genetics, starting with Frederick Griffith's transformation experiments in 1928 and culminating in the transformative molecular biology revolution of the 1970s.

Pre-Molecular Era (1928-1950)

- 1928 Frederick Griffith discovered bacterial metamorphosis
- 1944 Oswald Avery, Colin MacLeod, and Maclyn McCarty dug up DNA as principal substance of transmutation principle and took into light.
- 1950, Erwin Chargaff incorporated base- pairing rules
- 1952 Alfred Hershey and Martha Chase authenticated DNA as one transmissible evidence in biology field

Early Molecular Biology (1950-1970)

- 1953: Watson and Crick exposed the crude skeleton structure of DNA depicting authentic details before world.
- 1958: Central dogma theory constructed by Francis Crick
- 1961: The Triplet Code was deciphered by Marshall Nirenberg and Heinrich Matthaei.
- 1966: Entire genetic code formulated

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Resurgence frequency of >80% for plasmids from operable glycerol stocks

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Historical Timeline

The emergence of expression plasmids in the 1970s marked a pivotal transition from observational biology to hands-on molecular engineering, driven by several crucial breakthroughs. Among these, the discovery of restriction endonucleases by Arber, Smith, and Nathans, which garnered them the Nobel Prize in Physiology or Medicine in 1978, introduced indispensable gadgets for accurate DNA manipulation (Arber, 1965; Smith & Wilcox, 1970). Originally a part of bacterial defense mechanisms against invasive DNA, these enzymes provided scientists with precise "molecular scissors" to cut DNA at specific sequences, revolutionizing biological research.

Figure 1: Chronological model showing the development and key milestones in recombinant DNA technology and genetic engineering applications

Concurrently, the discovery and description of DNA ligase enabled DNA segments of different origins to be joined together, laying the foundation for recombinant DNA technology (Masamune & Richardson, 1968; Richardson et al., 1968). The enzyme's capacity to establish phosphodiester bonds between matching DNA ends empowered the creation of chimeric DNA molecules previously absent in nature.This capability was revolutionary, as it allowed researchers to combine genetic elements from disparate organisms, creating hybrid molecules with designed properties.

In 1973, Cohen and Boyer pioneered the creation of the first recombinant DNA molecules by successfully integrating foreign DNA into bacterial plasmids, as demonstrated through its stable maintenance and expression in Escherichia coli (Cohen et al., 1973). In their experiment, they transferred antibiotic resistance genes between plasmids, resulting in a chimeric plasmid that exhibited dual antibiotic resistance. Importantly, their work proved that foreign genes could be sustained within bacterial hosts and expressed as functional proteins, marking a significant milestone in genetic engineering. This breakthrough has made it feasible to produce hormones such as insulin, growth hormone, and interferons, previously obtainable only in limited supplies from natural sources, in virtually limitless quantities by harnessing engineered bacteria. This groundbreaking capability ignited the biotechnology revolution and facilitated the creation of pioneering companies such as Genentech, driving forward the commercialization of recombinant DNA technology with profound implications for science and industry.

Technological Infrastructure Development

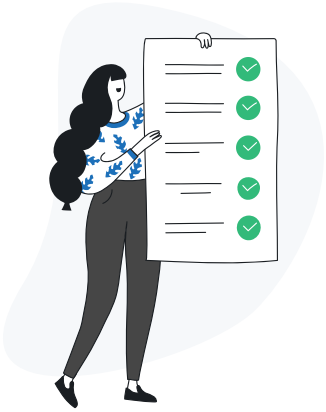
Following Cohen and Boyer's pivotal advancement, there was swift progress in developing the technological foundations essential for routine molecular cloning. Comprehensive restriction enzyme maps were constructed, efficient cloning protocols were developed, and standardized laboratory techniques were established, effectively transitioning molecular cloning from experimental novelty to routine practice in laboratories. The development of DNA sequencing techniques by Sanger and Gilbert, honored with the Nobel Prize in Chemistry in 1980, offered essential analytical tools for verifying and characterizing cloned sequences, highlighting the profound importance of these scientific

achievements. The ability to accurately read DNA sequences has been crucial in advancing the development of expression plasmids. This capability enables researchers to confirm the integrity of cloned genes and refine their expression by closely examining the sequences surrounding the genes of interest. This pivotal advancement in sequencing technology also enabled the thoughtful design of expression vectors with enhanced characteristics.

Evolution of Bacterial Expression Systems

The first expression plasmids were straightforward constructs that were modeled after naturally occurring bacterial plasmids like ColE1 and pBR322 (Bolivar et al., 1977). These innovative vectors marked the first concerted effort to develop standardized instruments for gene expression and molecular cloning. The pBR322 was the prototype for present time cloning vectors and was built by Francisco Bolivar and crew. It had necessary components, such as an origin of replication derived from ColE1, which allowed E to replicate autonomously. coli, two genes for antibiotic resistance (tetracycline resistance and ampicillin resistance) to select transformed cells, and distinct restriction enzyme sites for DNA insertion. These early vectors, however, lacked complex regulatory components necessary for regulated protein expression. The majority of gene expression was constitutive and reliant on either cryptic promoter activity within the vector sequences or weak prokaryotic promoters found in the cloned DNA fragments. When scientists tried to express proteins that were harmful to bacterial hosts or when high-level expression was necessary for protein purification and characterization, this limitation became evident.

The first systematic use of natural regulatory mechanisms to regulate the expression of recombinant genes was the lac promoter system, which represented a major breakthrough in bacterial expression technology. In their Nobel Prize-winning work, Jacob and Monod identified and described the lac operon, which offered a well-understood mechanism for lactose-inducible, glucose-repressible gene expression. The lac promoter-operator system and the lacZ α-complementation system for blue-white colony screening were integrated into the pUC series of vectors, which were created by Vieira and Messing (1982). This allowed for IPTG-inducible gene expression and simple recombinant clone identification. A number of innovations brought about by the pUC vectors have since become commonplace in expression vectors. Blue-white screen was made possible by the multiple cloning site (MCS), which had recognition sequences for a large number of restriction enzymes arranged to preserve the lacZ gene's reading frame. The high copy number (usually 500–700 copies per cell) that resulted from the replication origin of pMB1 offered a plentiful template for expression and cloning. In addition to being a selectable marker, the ampicillin resistance gene's small size (roughly 2 x 7 kb) allowed for effective transformation and manipulation. Growing bacterial cultures to high density prior to inducing protein expression was made possible by the lac system's strict regulation in the absence of an inducer and its potent induction upon the addition of IPTG. Known as "grow then induce," this method became a standard procedure that increased the yield of recombinant protein and bacterial biomass. Researchers found that the temperature, inducer concentration, timing of induction, and medium composition all had a significant impact on the yield and quality of proteins. IPTG concentrations were found to be optimal, but protein solubility was frequently enhanced by lower expression temperatures (15–25°C as opposed to 37°C).



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The shortcomings of first-generation vectors were addressed in the 1980s by the creation of increasingly complex bacterial expression systems. In order to achieve extraordinarily high levels of protein expression, the T7 expression system—which was developed by Studier and Moffatt (1986)—used the highly active T7 RNA polymerase and its cognate promoter. This system allowed for previously unheard-of levels of transcription from expression constructs because T7 RNA polymerase is both highly processive and specific for its promoter. The T7 system's elegant design was derived from its orthogonality to the transcriptional machinery of the host cell. Arised from bacteriophage T7, T7 RNA polymerase is judicious for T7 promoter sequences and is immune to the majority of E molecules. mechanisms that regulate E. coli. This lack of host regulation allows for steady, high-level expression irrespective of growth conditions or cellular metabolic state. Specialist E was needed for the system. coli strains C.

The development of BL21(DE3) and related strains represented a significant advance in host cell engineering. These strains were specifically designed for recombinant protein expression, incorporating features such as deficiency in the lon and ompT proteases to reduce protein degradation, and the DE3 prophage carrying the T7 RNA polymerase gene under lacUV5 control. The lacUV5 promoter variant provides tighter regulation than the wild-type lac promoter, reducing background expression of T7 RNA polymerase and therefore background expression of recombinant proteins.

The pET (plasmid for expression by T7 RNA polymerase) vector series became one of the most widely used expression systems for recombinant protein production in bacteria (Rosenberg et al., 1987). These vectors incorporated multiple innovative features that addressed common challenges in protein expression and purification. N-terminal His-tags, typically consisting of six consecutive histidine residues, enabled rapid protein purification using immobilized metal affinity chromatography (IMAC). Although the His-tag was a dependable purification handle, its tiny size and extreme affinity reduced interference with protein function. Additionally, the pET system introduced the idea of modular vector design, whereby various pET vectors are tailored for particular uses. Basic T7 expression was possible with pET-3 series vectors, but affinity tags and protease cleavage sites were added with pET-15b and later vectors. To solve the solubility problems with certain recombinant proteins, pET-22b incorporated a pelB signal sequence for periplasmic expression. Through this modular approach, researchers were able to choose vectors that were best suited to their particular needs for expression. The multiple cloning sites in pET vectors are intentionally designed to maintain correct reading frames and provide flexibility in cloning strategies. Increased mRNA stability and decreased read-through transcription that might disrupt host cell metabolism were the results of T7 transcription termination signals. Included in many pET are F1 origins.

Engineering Expression Hosts

The development of vectors was closely accompanied by a major effort to engineer bacterial host strains that were best suited for the expression of recombinant proteins. Although many variations of

BL21 derivatives were created for particular uses, these became the industry standard for expression hosts. In order to address issues with toxic protein expression, Miroux and Walker created the C41(DE3) and C43(DE3) strains, which included mutations that decreased the intensity of T7 expression. In typical BL21(DE3) hosts, these strains allowed the expression of proteins that were fatal. Because of the more oxidizing cytoplasmic environment that the origami strains with thioredoxin reductase and glutathione reductase mutations offered, disulfide bond formation in recombinant proteins was encouraged. The issue of tRNA availability for rare codons was resolved by the creation of strains that were optimized for codons. Plasmids encoding tRNAs for uncommon codons (AGA, AGG, AUA, CUA, CCC, and GGA) added to Rosetta strains permitted eukaryotic G to be expressed.

Specialized Bacterial Expression Features

The creation of fusion protein systems, which represent an advanced method of getting around the drawbacks of direct protein expression, addressed basic issues with protein solubility, stability, and purification. By offering a highly specific affinity purification system in addition to a solubility enhancement tag, the glutathione S-transferase (GST) fusion system, first presented by Smith and Johnson in 1988, completely changed protein purification. The 26 kDa enzyme GST, which is found in Schistosoma japonicum, has a stable tertiary structure, high solubility in aqueous solutions, and high-affinity binding to glutathione-conjugated resins, all of which make it a perfect fusion partner. The pGEX vector series incorporates the GST gene solely directed by the tac promoter, enabling the IPTG-inducible expression of GST fusion proteins (Borgi & Gargouri, 2008). The system's elegance lay in its simplicity—crude bacterial lysates could be applied directly to glutathione-Sepharose columns, with fusion proteins binding specifically while most bacterial proteins flowed through unbound. Employing elution with reduced glutathione effectively produced purified fusion proteins through a single chromatographic procedure, significantly minimizing the duration and complexity associated with protein purification protocols. Maltose-binding protein (MBP) fusions, created by di Guan and associates in 1988, also offered an extra purification handle and greatly increased the solubility of recombinant proteins. The 42 kDa periplasmic protein MBP is detected in E. coli, which frequently makes it possible to produce soluble proteins that would otherwise form inclusion bodies, has the remarkable ability to increase the solubility of fused proteins. Clone sites were positioned to produce in-frame fusions when the malE gene encoding MBP was integrated into the pMAL vector system, which was controlled by the ptac promoter. In addition to enhancing solubility, the MBP system provided several other advantages. Fusion proteins could be guided to the periplasmic space, where the oxidizing environment promoted appropriate disulfide bond formation and where many bacterial proteases were lacking, by including the natural signal sequence. The use of amylose resin, which bound MBP with great affinity and specificity, allowed for purification. Additionally, the system is installed.

Thioredoxin (Trx) fusion systems addressed specific challenges associated with the expression of proteins containing disulfide bonds. Thioredoxin, a small redox-active protein, not only enhanced protein solubility but also facilitated proper disulfide bond formation through its intrinsic oxidoreductase activity. The pET-32 system combined thioredoxin with His-tag and S-tag elements, creating a well-equipped fusion system that addressed multiple expression and purification challenges simultaneously.

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Plasmids encoding tRNAs for uncommon codons (AGA, AGG, AUA, CUA, CCC, and GGA) added to Rosetta strains permitted eukaryotic G to be expressed.

https://pubmed.ncbi.nlm.nih.gov/16703415

The pGEX vector series incorporates the GST gene solely directed by the tac promoter, enabling the IPTG-inducible expression of GST fusion proteins (Borgi & Gargouri, 2008).

https://pubmed.ncbi.nlm.nih.gov/18434194

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Advanced Mammalian Expression Features

The development of internal ribosome entry sites (IRES) enabled the expression of multiple proteins from a single transcript, facilitating the co-expression of proteins and selection markers (Jang et al., 1988). Self-cleaving peptide sequences, such as 2A peptides, provided an alternative approach for polycistronic expression with more predictable stoichiometry (Ryan et al., 1991). Tissue-specific and cell-type-specific promoters were incorporated into mammalian expression vectors to achieve targeted expression patterns. For instance, the muscle-specific promoters such as muscle creatine kinase and myosin heavy chain, alongside neuron-specific promoters like synapsin and neurofilament, facilitated research focused on expression patterns specific to particular cell types (Rindt et al., 1993).

Modern Developments and Synthetic Biology

a) Synthetic promoters, designed with specific regulatory elements, offer precise control over gene expression in terms of levels, timing, and cellular specificity (Redden & Alper, 2015). These engineered systems often incorporate multiple regulatory inputs, enabling complex expression logic circuits.

b) The adaptation of CRISPR-Cas technology for transcriptional activation has created new paradigms for expression control. dCas9-based activator systems (CRISPRa) enable programmable gene activation through guide RNA targeting, providing flexible and multiplexable expression control (Gilbert et al., 2013). These systems offer advantages in terms of modularity and the ability to simultaneously regulate multiple genes.

c) Cell-free protein synthesis systems have increasingly become viable alternatives to traditional cell-based expression methods, which hold particular advantages in handling toxic proteins, enabling swift prototyping, and facilitating the integration of non-natural amino acids. Both prokaryotic and eukaryotic cell-free systems have been developed, with recent advances improving yield, reaction duration, and protein quality (Carlson et al., 2012).

Plasmid Degradation and Restoration Techniques

Plasmid degradation represents a significant challenge in both laboratory settings and clinical applications. The degradation can occur through multiple pathways, including enzymatic cleavage by nucleases, oxidative damage from reactive oxygen species, thermal denaturation, and physical shearing during handling and storage (Molina, 2021). These degradation mechanisms can lead to linearization of circular plasmids, introduction of nicks and breaks, base modifications, and complete fragmentation of the DNA molecule. The expanding applications of plasmids heighten the importance of developing effective restoration techniques. While conventional methods have primarily aimed at preventing degradation by ensuring optimal storage conditions and applying protective additives, recent scientific progress has ushered in innovative enzymatic repair systems, chemical restoration techniques, and biotechnological strategies designed to actively mend damaged plasmid DNA (Sun et al., 2019).

Mechanisms of Plasmid Degradation

Nuclease-Mediated Degradation

Nucleases pose a significant threat to the integrity of plasmids, especially in samples contaminated with cellular debris or not properly purifie (Green & Sambrook, 2012). These enzymes are fundamentally categorized as endonucleases, responsible for cleaving internal phosphodiester bonds, thereby inducing breaks that convert supercoiled plasmids into relaxed or linear configurations (Linn & Roberts, 1982), and Exonucleases operate by excising nucleotides from the terminal sections of DNA. The functioning of these nucleases is subject to a myriad of factors such as pH, temperature, ionic strength, and notably, the presence of divalent cations like Mg^{2+} and Ca^{2+} (Lazarus & Seaman, 2021). DNase I, in particular, necessitates Mg^{2+} or Mn^{2+} to achieve peak activity, and it operates effectively at physiological pH levels, highlighting its potential disruptive impact in biological systems.

Table 1: Classification of Nucleases Affecting Plasmid Stability

Oxidative Degradation

Reactive oxygen species (ROS) pose a significant threat to plasmid DNA through multiple oxidative pathways. Reactive oxygen species, notably affecting DNA, include hydroxyl radicals(•OH), superoxide anions ($O_2•^-$), hydrogen peroxide (H_2O_2), and singlet oxygen($^1O_2$) (Cadet et al., 2010). The oxidative damage manifests in several forms:

1. Base Modifications: The most common oxidative lesion is 8-oxo-7,8-dihydroguanine (8-oxoG), formed through guanine oxidation. This modification can lead to G:C to T:A transversion mutations during replication (Halliwell & Gutteridge, 2015). The accumulation of such lesions over extended storage periods can lead to replication errors, strand breaks, and ultimately complete loss of plasmid functionality (Cadet et al., 2003).
2. Sugar-Phosphate Backbone Damage: Oxidation of the deoxyribose sugar can result in strand breaks, with the C1' and C4' positions being particularly susceptible to abstraction reactions.
3. Cross-linking: Bifunctional oxidants can create interstrand or intra-strand cross-links, severely compromising DNA structure and function.

The kinetic parameters for oxidative damage vary significantly based on the oxidant species and environmental conditions.

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These enzymes are fundamentally categorized as endonucleases, responsible for cleaving internal phosphodiester bonds, thereby inducing breaks that convert supercoiled plasmids into relaxed or linear configurations (Linn & Roberts, 1982),
https://bio.libretexts.org/Bookshelves/Biotechnology/Bio-OER_(CUNY)/08%253A_Analyzing_DNA/8.05%253A_Restriction_Enzymes

Table 1: Classification of Nucleases Affecting Plasmid Stability
https://pmc.ncbi.nlm.nih.gov/articles/PMC4071455

Reactive oxygen species, notably affecting DNA, include hydroxyl radicals(•OH), superoxide anions ($O_2•^-$), hydrogen peroxide (H_2O_2), and singlet oxygen($^1O_2$) (Cadet et al., 2010).

https://vmi.pitt.edu/research/cores/epr-ros

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Thermal Degradation

Thermal stress affects plasmid stability through multiple mechanisms, including DNA melting, depurination, and acceleration of chemical degradation reactions. Plasmids exhibit thermal stability, which is defined by their melting temperature (Karp et al.), which is influenced by various factors, including the guanine-cytosine (G-C) content, ionic strength, and pH levels. (Jin et al., 2018).

The Arrhenius equation describes the temperature dependence of degradation rates:

k = A × e^(-Ea/RT)

Where k denotes the rate constant, A symbolizes the pre-exponential factor, Ea depicts the activation energy, R is the gas constant, and T signifies the absolute temperature. Depurination represents a particularly significant thermal degradation pathway, with an activation energy of approximately 30 kcal/mol for the glycosidic bond cleavage (Lindahl, 1974). This process generates apurinic (Qing et al.) sites that are highly unstable and prone to strand breakage.

Mechanical Degradation

Plasmid degradation can occur due to physical forces such as shear stress, cavitation, and compression. The forces in question are frequently observed in activities such as pipetting, vortexing, sonication, as well as during freeze-thaw cycles.

The relationship between shear rate and DNA degradation follows the relationship:

$\tau = \eta \times \dot{\gamma}$

Where τ denotes the shear stress, η is the fluid viscosity, and $\dot{\gamma}$ is the shear rate.

High molecular weight DNA, including supercoiled plasmids, is particularly susceptible to mechanical degradation due to the increased hydrodynamic stress on longer molecules.

Hydrolytic Damage

Under physiological conditions, continuous hydrolytic damage arises from the spontaneous breakdown of phosphodiester bonds and glycosidic linkages, a process exacerbated by increased temperatures, extreme levels of pH, and the presence of divalent metal ions (Friedberg et al., 2006).

Spontaneous Hydrolysis:

Reaction Mechanism: $R\text{-}PO_4^-\text{-}R' + H_2O \rightarrow R\text{-}PO_4H_2 + R'\text{-}OH$

Rate Factors:

- Temperature: It doubles with every 10°C rise in temperature
- pH: Maximum stability at pH 7.0-8.0
- Ionic strength: Metal ions catalyse hydrolysis
- Time: First-order kinetics for backbone cleavage

Quantitative Degradation Kinetics:

• Half-life at 25°C, pH 7.0: Approximately 30,000 years
• Half-life at 95°C, pH 7.0: Estimated to be approximately 30 minutes
• Temperature coefficient: For hydrolysis reactions, the coefficient, Q_{10} = 2-3

• pH sensitivity: 10-fold rate increase with each pH unit above 8.0

Storage Conditions and Stability Factors

The stability of plasmid DNA during storage is a matter of utmost importance, as it hinges on several critical environmental factors such as temperature, humidity, the composition of buffers, and the materials used for containers (Anchordoquy & Molina, 2007). For long-term preservation, storage at -80°C with suitable buffers generally ensures remarkable stability extending over years or even decades. Conversely, storage at 4°C poses a risk of gradual degradation over months to years, which largely depends on the conditions of the buffer (Bonner & Laskey, 1974). The choice of storage buffer significantly impacts plasmid stability, with Tris-EDTA (TE) buffer providing protection against both nuclease activity and metal-catalyzed oxidation through pH buffering and metal chelation respectively (Green & Sambrook, 2012). The addition of antioxidants like ascorbate or reduced glutathione is crucial in safeguarding against oxidative damage, especially concerning long-term storage needs (Heller, 1992). The selection of container materials and the conditions in which storage occurs play a pivotal role in affecting plasmid stability due to interactions with plastic surfaces, exposure to UV light, and potential contamination from atmospheric pollutants (Anchordoquy & Molina, 2007). Implementing low-binding plasticware and utilizing amber-colored containers are important measures to reduce surface interactions and prevent light-induced damage (Huber et al., 1993).

Plasmid Restoration Techniques

Plasmid restoration necessitates the deployment of various systems, determined by the extent and nature of the degradation observed. For minor degradation, it is often sufficient to carry out simple re-transformation into suitable bacterial hosts, followed by thorough colony screening and plasmid preparation. However, more significant degradation may require the use of molecular reconstruction techniques, such as PCR amplification of specific regions or even the complete re-synthesis of the plasmid (Green & Sambrook, 2012). Quality control measures for restored plasmids typically include restriction enzyme analysis, PCR verification of key regions, and complete sequencing of critical elements. Next-generation sequencing technologies have revolutionized plasmid verification by enabling rapid, comprehensive sequence confirmation at reduced costs (Goodwin et al., 2016). As repositories grow increasingly in both size and complexity, the establishment of standardized quality metrics for plasmid collections is essential. These metrics commonly encompass transformation efficiency, the accuracy of sequence fidelity, and thorough functional validation achieved through expression studies (Cozzi et al., 2017).

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Where k denotes the rate constant, A symbolizes the pre-exponential factor, Ea depicts the activation energy, R is the gas constant, and T signifies the absolute temperature.
https://www.solubilityofthings.com/arrhenius-equation-and-its-significance-0

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Enzymatic Restoration Systems

Enzymatic restoration represents the most sophisticated approach to plasmid repair, utilizing the cell's natural DNA repair machinery or engineered enzyme systems.

DNA Ligase-Based Restoration

T4 DNA Ligase: This enzyme exhibits broad substrate specificity and can ligate both cohesive and blunt-ended DNA fragments. The ideal reaction conditions involve maintaining a temperature of 16°C for a duration of 4 to 16 hours, while ensuring the presence of ATP and Mg^{2+} ions.

E. coli DNA Ligase: An enzyme reliant on NAD^{+} predominantly enables precise joining of cohesive DNA termini.

The ligation efficiency can be calculated this way :

Efficiency = (Ligated molecules / Total molecules) × 100%

Polymerase-Based Restoration

DNA polymerases have the capacity to rectify damaged plasmids by employing their 3' to 5' exonuclease activity, which serves as a proofreading mechanism allied with their polymerase activity.

Key enzymes include:

T4 DNA Polymerase: Possesses strong 3' to 5' exonuclease activity, making it effective for removing damaged bases and filling in gaps.

Pfu DNA Polymerase: High-fidelity enzyme with proofreading capability, suitable for accurate restoration of coding sequences.

Klenow Fragment: Modified E. coli DNA Polymerase I lacking 5' to 3' exonuclease activity, useful for fill-in reactions.

Multi-Enzyme Restoration Systems

Complete restoration often requires multiple enzymatic activities working in concert.

Figure 2: Schematic representation of multi-enzyme plasmid restoration pathway showing the sequential action of nucleases, polymerases, and ligases.

Chemical Restoration Methods

Chemical approaches to plasmid restoration focus on neutralizing damaging agents and providing chemical environments that favor DNA stability.

Antioxidant Systems

Antioxidants prevent and reverse oxidative damage through various mechanisms:

Reducing Agents:

- Dithiothreitol (DTT): Reduces disulfide bonds and chelates metal ions
- β-Mercaptoethanol: Similar reducing properties with lower toxicity
- Tris(2-carboxyethyl)phosphine (TCEP): Non-thiol reducing agent

Radical Scavengers:

- Mannitol: Scavenges hydroxyl radicals with high efficiency

• Dimethyl sulfoxide (DMSO): Broad-spectrum radical scavenger
• Ascorbic acid: Water-soluble antioxidant effective against multiple ROS
Metal Chelators:
• EDTA: Chelates bind divalent cations, effectively inhibiting oxidation catalyzed by metals.
• Desferrioxamine: Specific iron chelator
• Bathocuproine: Copper-specific chelator
pH Buffering Systems
• Maintaining optimal pH is crucial for plasmid stability, as extreme pH values can cause base deprotonation and strand separation. Common buffering systems include:
Table 3: Buffering Systems for Plasmid Stabilization

Physical Restoration Approaches
Physical techniques aimed at plasmid restoration concentrate on fine-tuning environmental parameters and applying physical forces to ensure the correct configuration of DNA, underscoring the critical nature of these processes.
Thermal Annealing
Controlled thermal cycling can restore hydrogen bonding in partially denatured plasmids. The annealing process follows the relationship:
Rate of annealing $\propto \sqrt{C_0 t}$; (Where C_0 is the initial DNA concentration and t is time)
Optimal annealing conditions typically involve:
• Temperature: 65-68°C (5°C below Tm)
• Time: 30-60 minutes
• Salt concentration: 150-500 mM NaCl
Electrophoretic Restoration
Pulsed-field gel electrophoresis (PFGE) is a sophisticated method specifically crafted to effectively separate and recover intact plasmids from mixtures that contain fragmented or degraded DNA. The separation is based on size-dependent mobility differences under alternating electric fields.
Osmotic Stabilization
Osmotic agents help maintain DNA structure by controlling water activity and preventing excessive hydration or dehydration:
 Polyethylene glycol (PEG): Molecular crowding agent that stabilizes DNA structure
 Trehalose: Disaccharide that forms hydrogen bonds with DNA
 Glycerol: Cryoprotectant that maintains DNA flexibility at low temperatures

Comparative Analysis of Restoration Techniques
Efficacy Comparison
The effectiveness of different restoration techniques varies significantly based on the type and extent of damage.
Table 4: Assessing the Relative Effectiveness of Plasmid Restoration Methods
Cost-Benefit Analysis
Economic considerations are crucial for selecting appropriate restoration techniques:
Table 5: Cost-Benefit Analysis of Restoration Methods
Cost-Effectiveness Index = Success Rate / (Cost × Time)

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The ligation efficiency can be calculated this way :
https://theory.labster.com/ligation

Rate of annealing $\propto \sqrt{C_0 t}$; (Where C_0 is the initial DNA concentration and t is time)
https://nij.ojp.gov/nij-hosted-online-training-courses/dna-amplification/overview/thermal-cycling-parameters-optimization

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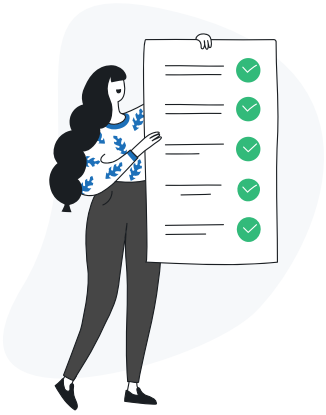
The systematic cataloging of plasmid collections demands a standardized methodology that meticulously records crucial information yet remains sufficiently adaptable to encompass various vector types and applications (Karp et al., 2009). Implementing hierarchical classification systems, defined by vector backbone, insert type, and intended application, ensures an intuitive structure that significantly enhances researchers' abilities to search and browse efficiently (Miller & Calos, 2007). The development of standardized nomenclature systems enables consistent identification and communication regarding specific plasmid constructs across different laboratories and research institutions (Canton et al., 2008). The Registry of Standard Biological Parts represents one example of such standardization efforts, providing unique identifiers and standardized descriptions for genetic components used in synthetic biology applications. The design of a database schema must thoughtfully address the intricate relationships among plasmids, their constituent parts, and the associated experimental data, ensuring efficient query capabilities and maintaining data integrity constraints (Garcia-Hernandez et al., 2002). In essence, relational database models systematically arrange information into interconnected tables that represent plasmids, sequences, features, publications, and experimental results, reflecting the complexity and importance of these components (Karp et al., 2010). Molecular Barcoding and Tracking Systems Molecular barcoding systems provide a reliable method for plasmid identification, ensuring precise results irrespective of label condition and reducing human errors in record keeping (Brenner & Lerner, 1992). DNA barcoding techniques utilize distinct sequence identifiers integrated into plasmid constructs, allowing them to be swiftly sequenced or identified using PCR-based methods to verify plasmid identity (Hebert et al., 2003). The integration of barcoding systems with laboratory automation enables high-throughput processing and tracking of plasmid samples throughout preparation, storage, and distribution workflows. Robotic systems possess the capability to autonomously sequence barcode regions and update database records, significantly reducing the risk of sample mix-ups and ensuring precise inventory control (Dove, 1999). Two-dimensional barcode systems, like Data Matrix codes, are adept at encoding extensive data within compact formats, making them ideal for labeling small sample containers while remaining easily readable by conventional laboratory equipment (Kato & Tan, 2005). The implementation of these systems alongside laboratory information management systems (LIMS) facilitates thorough tracking of sample history and experimental usage, underscoring their critical role in laboratory operations (Thoma et al., 2001). Digital Storage and Retrieval Systems Digital storage systems for plasmid information necessitate a thoughtful balance between accessibility, security, and long-term preservation, addressing the varied needs of research communities. While cloud-based platforms offer scalability, automatic backups, and multi-site accessibility, it is imperative to meticulously evaluate data security and ownership concerns (Armbrust et al., 2010). Moreover, the adoption of version control systems from software development practices serves as a crucial method for meticulously tracking changes in plasmid sequences and their associated documentation over time (Chacon &

Straub, 2014). It is imperative that search and retrieval algorithms are designed to accommodate the varied approaches researchers use to query plasmid databases, such as sequence-based searches, feature-oriented questions, and literature-associated inquiries (Altschul et al., 1990). The implementation of full-text indexing and semantic search capabilities can significantly enhance the utility of plasmid databases for research applications (Manning et al., 2008) Quality Assurance and Standardization Analytical Methods for Plasmid Characterization Plasmid in-depth characterization involves the implication of various methodological approaches and evaluation criteria focusing on structural integrity, purity, and function (Joseph Sambrook, 2012). Spectrophotometric analysis provides rapid assessment of DNA concentration and purity through measurement of absorbance at 260 nm and calculation of A260/A280 and A260/A230 ratios (Joseph Sambrook, 2012). Pure DNA preparations typically exhibit A260/A280 ratios between 1.8 and 2.0, with lower values indicating protein contamination and higher values suggesting RNA contamination or alkaline conditions. Fluorometric measurement methods, using DNA-specific fluorescent dyes (e.g., PicoGreen or SYBR Green) have a higher sensitivity and specificity than spectrophotometric methods and especially are useful for low-content samples . These procedures are less sensitive to contamination and more specifically quantitate in the presence of RNA or protein impurities (Ahn et al., 1996). Capillary electrophoresis systems offer high-resolution analysis of plasmid preparations, enabling the detection and quantification of different topological forms including supercoiled, relaxed, and linear DNA (Hebenbrock et al., 1995). Automated capillary electrophoresis systems can process multiple samples rapidly while providing quantitative data on the relative proportions of different plasmid forms (Birren & Lai, 1994) Contamination Detection and Prevention Contamination in plasmid preparation and storage poses a serious and ongoing challenge, with possible sources being bacterial chromosomal DNA, RNA, proteins, endotoxins, and foreign plasmids (Joseph Sambrook, 2012). Bacterial chromosomal DNA contamination can be assessed through restriction enzyme analysis using enzymes that cut chromosomal DNA frequently but do not cut the plasmid of interest (Birnboim & Doly, 1979). RNA contamination can be detected through agarose gel electrophoresis and quantified using RNase treatment followed by spectrophotometric analysis. Plasmid preparation procedures that include RNase treatment effectively remove RNA contamination while retaining plasmid DNA (Birnboim & Doly, 1979). Endotoxin contamination is of particular importance for plasmids for use in mammalian cell culture or in mammalian in vivo applications, as extremely low levels can result in inflammatory responses and confound experiments. Limulus amebocyte lysate (LAL) assays provide sensitive detection of endotoxin contamination, with chromogenic and fluorogenic variants enabling quantitative analysis (Williams, 2019). Database Architecture and Design Principles This database has experienced several developmental stages in the progression of plasmid databases. Earlier systems like Plasmid Database at University of Wisconsin were more specialized, and were primarily focused on sequence storage, with manual annotation. With the genomics revolution that took place in early 2000s, more elaborate systems have been designed. The ACLAME database A Classification of Mobile genetic Elements introduced some of the first systematic classification of mobile genetic elements, such as plasmids, and set important standards in annotation (Leplae et al., 2010). This era also witnessed the introduction of webforms, and the use of standardised data types like GenBank. Current plasmid databases have adopted high-throughput data processing and automated analysis pipelines. Systems, such as PLSDB and PlasmidFinder, are included in the current state of art and have implemented high-level sequence analysis algorithms and extensive web-based analysis tools (Carattoli et al., 2014; Galata et al., 2018) The high



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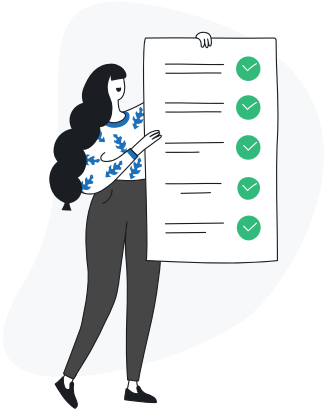
NoSQL Database Approaches Document-oriented databases like MongoDB are increasingly adopted for managing plasmid data, especially where flexible schema design and horizontal scaling are needed. The document model effectively supports the hierarchical structure of genomic annotations and facilitates the efficient storage of diverse metadata formats (Garcillán-Barcia et al., 2009). Notable advantages of NoSQL approaches include schema flexibility, allowing for dynamic adaptation to evolving data formats and annotation standards. Native JSON functionality enhances data exchange with web-based applications and bioinformatics platforms. The ability to scale horizontally allows for distribution across multiple servers, improving execution speed for extensive analytical tasks. Graph databases, such as Neo4j and Amazon Neptune, are increasingly adopted for managing plasmid data due to their ability to effectively depict intricate evolutionary ties and networks of sequence similarity. These databases offer specialized graph query languages refined for navigating the complex relational constructs typical in comparative genomics studies. Hybrid Architectures Several modern plasmid databases use hybrid architectures that integrate various database technologies to enhance performance for diverse data types and query patterns. Such designs commonly incorporate a relational database for structured metadata and sequence identifiers, alongside NoSQL systems for flexible annotation data, and graph databases for mapping relationships. These hybrid approaches allow for the optimization of distinct components tailored to specific use cases, while ensuring system coherence through clearly defined APIs and data synchronization protocols. Nevertheless, they also add layers of complexity concerning system administration, data consistency management, and query optimization across various platforms. Major Plasmid Database Systems PLSDB (Plasmid Database) PLSDB serves as one of the most extensive modern plasmid databases, encompassing more than 15,000 complete plasmid sequences with standardized metadata and automated annotation processes (Galata et al., 2018). It operates on a PostgreSQL backend, enhanced with custom indexing optimizations tailored for genomic data. The database's architecture integrates numerous advanced features, including automated quality control workflows, standardized systems for taxonomic classification, and detailed profiling of resistance genes. The annotation pipeline incorporates a suite of advanced prediction tools, such as Prokka for gene prediction, BLAST for conducting homology searches, and various specialized tools for the detection of mobile elements. PLSDB's indexing strategy combines traditional B-tree indexes for metadata queries with custom sequence indexes optimized for similarity searching. The web interface offers advanced search capabilities, including sequence similarity searches, metadata filtering, and batch download options. Additionally, RESTful APIs facilitate programmatic access for high-throughput applications and integration with third-party analytical tools. PlasmidFinder Database PlasmidFinder is a specialized tool dedicated to the identification and typing of plasmids via a meticulously curated database of plasmid replicon sequences (Carattoli et al., 2014). The system is designed with an efficient architecture that enhances rapid sequence classification and facilitates real-time analysis through web-based applications. At its

core, the database comprises thoughtfully curated replicon sequences that represent key plasmid incompatibility groups. Each sequence is linked to detailed metadata, encompassing incompatibility group classifications, host range specifics, and relevant literature references. Domain experts manually conduct the curation process to ensure the reference sequences are of the highest quality. The analytical pipeline utilizes BLAST-based similarity searches with meticulously optimized parameters for effective plasmid classification. Integration with whole-genome sequencing workflows facilitates the automatic detection of plasmids within bacterial genome assemblies. The web interface of PlasmidFinder is carefully designed to be user-friendly, providing efficient input methods and presenting results in a clear and concise manner. The system accommodates both individual sequence queries and batch processing to support high-throughput applications. COMPASS (Comparative Plasmid Suite) COMPASS (Comparative Plasmid Suite) COMPASS offers an extensive platform for plasmid comparison and analysis, integrating various database backends and specialized analytical tools . Designed with a microservices architecture, the system allows for modular deployment and tailored optimization across different analytical capacities. The database component is built on a hybrid architecture that merges relational and NoSQL technologies, with primary sequence and metadata housed in PostgreSQL databases, while dynamic annotation data and analytical outcomes are stored in MongoDB collections. This method allows for optimization tailored to various query patterns while preserving the system's overall coherence. It offers support for multiple sequence alignment algorithms and includes integrated visualization tools for conducting comparative analyses. Established Indexing Strategies and Algorithms Sequence-Based Indexing 1. k-mer Indexing Approaches k-mer indexing has emerged as a fundamental technique for rapid sequence similarity searching in plasmid databases. This approach decomposes sequences into overlapping substrings of fixed length k, enabling efficient approximate matching and classification (Lock et al., 2018). The selection of the k-value is a crucial optimization parameter, wherein lower values enhance sensitivity but compromise specificity. Advanced k-mer indexing systems such as Kraken2 employ taxonomic classification trees to enable rapid species-level identification of query sequences. The system efficiently creates a concise hash table that associates k-mers with taxonomic identifiers, allowing the classification of millions of sequences per hour using standard hardware (Lock et al., 2018). MinHash algorithms offer a different method for k-mer indexing by generating compact sketches that maintain sequence similarity relationships and significantly decrease storage needs. Tools like Sourmash employ MinHash sketching to conduct comprehensive sequence comparisons, enabling effective clustering and similarity searches across large collections of sequences (Brown & Irber, 2016). 2. Suffix Arrays and Suffix Trees Suffix arrays and suffix trees provide exact pattern matching capabilities essential for identifying conserved sequences and regulatory elements within plasmid collections. These data structures enable efficient identification of a given pattern within a sequence database, with query times proportional to the pattern length rather than the database size (Gusfield, 1997). Contemporary methods for constructing suffix arrays utilize space-efficient algorithms and compressed formats to reduce memory usage. The Burrows-Wheeler Transform offers a notably effective technique for developing compressed suffix arrays, making it well-suited for extensive genomic databases. Feature-Based Indexing 1. Gene Annotation Indexing The comprehensive indexing of functional annotations facilitates complex queries that integrate sequence similarity with functional attributes. Contemporary plasmid databases possess specialized indexes for various annotation categories, such as antibiotic resistance genes, virulence factors, mobile genetic elements, and metabolic pathways. The ResFinder database serves as a prime example of advanced annotation indexing, as it maintains curated collections of antibiotic resistance genes



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Structural Feature Indexing

Plasmid structural features including origin of replication sequences, partition systems, and conjugation apparatus represent important indexing targets for specialized analyses. Despite overall divergence in sequence, these features exhibit conservation, requiring the implementation of specialized indexing strategies. Hidden Markov Models (HMMs) deliver efficient instruments for indexing structure features defined by sequence motifs with variable conservation patterns. Databases like Pfam offer pre-curated HMM libraries for protein families typically linked with plasmid functions (Finn et al., 2016).

3. Metadata Indexing

Contemporary systems use multi-dimensional indexing models that support various types of metadata such as taxonomic classifications, sources of isolation, geographic origins, and experimental conditions. These strategies often utilize ontology-based indexing approaches that apply standardized vocabularies, thereby facilitating semantic queries across diverse metadata collections. The Gene Ontology serves as a hierarchical classification system delineating biological processes, molecular functions, and cellular components (Gene Ontology Consortium, 2019). The integration of ontological indexing allows for advanced queries, including the identification of plasmids that contain genes participating in particular metabolic pathways or stress responses.

Data Integration and Standardization

Ontology Integration

The Gene Ontology presents a structured approach to functional annotation, enabling the systematic classification of functions encoded by plasmids, as outlined by the Gene Ontology Consortium in 2019 (Ferrer-Miralles et al.). Concurrently, the Antibiotic Resistance Ontology offers a specialized lexicon for understanding resistance mechanisms, thereby allowing precise classification of resistance determinants and associated phenotypes (Jia et al., 2016). This ontological structure supports complex queries that integrate genetic and phenotypic data, thus enabling exhaustive profiling of resistance.

The Sequence Ontology (SO) establishes standardized terminology for genomic features to ensure consistent annotation frameworks across various database systems (Eilbeck & Lewis, 2004). By integrating SO terms, automated annotation pipelines gain interoperability, enhancing data integration across numerous platforms.

Data Format Standardization

Standardized data formats are essential for achieving interoperability among various plasmid database systems. The GenBank format continues to be the leading standard for exchanging sequence data, offering a well-organized depiction of sequences along with their associated annotations (Benson et al., 2018). Meanwhile, the Generic Feature Format version 3 (GFF3) provides a more adaptable framework for representing genomic annotations, fostering hierarchical feature relationships and supporting systems with extensible attributes (Sequence Ontology Project, 2016). To enhance

interoperability, many modern systems are compatible with both GenBank and GFF3 formats. JSON-LD, short for JavaScript Object Notation for Linked Data, is increasingly recognized as a standard format for representing interconnected biological data. Its adoption enhances the integration of semantic web technologies and promotes automated data discovery, as highlighted by Sporny and colleagues in 2014. This format supports assimilation into larger scientific data ecosystems, permitting automated discovery of relationships across various data types.

API Development and Interoperability

RESTful APIs are crucial elements in contemporary plasmid databases, facilitating programmatic access and integration with analytical pipelines. They usually offer endpoints for retrieving sequences, querying metadata, and accessing analytical services, adhering to standard HTTP protocols to ensure a uniform interface design (Fielding, 2000). The Bioschemas initiative promotes standardized schemas for representing biological data on the web, thus enhancing automated data discovery and integration. The Bioschemas markup implemented in plasmid databases facilitates search engine indexing and the automated harvesting of the data for meta-analyses. GraphQL is an emerging alternative to REST APIs, offering more flexible querying possibilities and allowing clients to only retrieve the data needed for their analyses. Several bioinformatics platforms have started adopting GraphQL for its superior performance character and developer's experience.

Machine Learning Integration

Automated Classification Systems

Machine learning algorithms have transformed plasmid classification and annotation by facilitating the automated analysis of large-scale sequence data. Support Vector Machines (SVMs) have been especially successful at predicting plasmid hosts, with accuracy rates over 90% on various training datasets (Arredondo-Alonso et al., 2017). Random Forest algorithms provide excellent classification capabilities for complex multi-class challenges, such as predicting plasmid mobility. These ensemble techniques blend several decision trees to produce high-accuracy predictions with interpretable feature importance rankings that uncover the sequence features most informative of certain phenotypes (Jin et al., 2018). Neural network methodology, especially deep learning topologies, also have been successful in automated plasmid annotation and functional prediction. Convolutional Neural Networks (CNNs) are capable of recognizing sequence motifs and structural motifs that are significant for plasmid function, whereas Recurrent Neural Networks (RNNs) are particularly good at handling variable-length sequence data (Asgari & Mofrad, 2015).

Similarity Clustering

Advanced clustering algorithms enable systematic organization of plasmid collections based on sequence similarity, functional content, and evolutionary relationships. Hierarchical clustering techniques offer tree-based intuitive models of sequence relationships, while k-means clustering offers computationally optimal techniques for partitioning large-scale data. UMAP and t-SNE offer computationally efficient dimensionality reduction techniques for visualization of high-dimensional sequence data in two or three dimensions (McInnes et al., 2018). These techniques offer the capability to detect sequence clusters and outliers that could be indicative of new plasmid families or sequencing errors. Graph-based clustering techniques like Leiden clustering offer high performance for network data, allowing identification of highly connected sequence clusters without sacrificing computational efficiency for large-scale data.

Machine learning algorithms allow prediction of plasmid properties from sequence data only, reducing dependence on experimental validation for first-order predictions of function. Host range prediction algorithms combine sequence properties with known associations to predict prospective bacterial hosts for new plasmid sequences (Arredondo-Alonso et al., 2017). Prediction of conjugation potential is another important application, with algorithms trained on representative conjugative and non-conjugative plasmids precisely predicting transfer ability from sequence content (Smillie et al., 2010). Prediction is particularly valuable for prediction of horizontal gene transfer capacity in environmental communities. Stability prediction algorithms predict plasmid maintenance traits from presence and arrangement of replication and partition systems. These algorithms provide insight into plasmid ecology and evolution processes, guiding plasmid persistence research in bacterial populations.

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JSON-LD, short for JavaScript Object Notation for Linked Data, is increasingly recognized as a standard format for representing interconnected biological data.

https://json-ld.org

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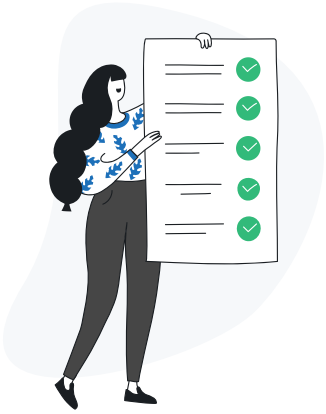
Quality Control and Validation Data Validation Frameworks Plasmid databases can only be efficient when comprehensive multidisciplinary quality control systems have been implemented. An automated validation pipeline checks the quality of the sequences with respect to assembly completeness, level of contamination, and consistency of annotations. There is a remarkable quality that is provided within genomes and plasmid assemblies which is based on single-copy gene presence and contamination analysis through CheckM (Parks et al., 2015). The system also detects possible chimeric sequences and assembly errors, which can be critical for many downstream analyses. Prokka also provides a standard annotation pipeline PGAP (Prokaryotic Genome Annotation Pipeline) that also incorporates automated quality control operational procedures (Seemann, 2014; Tatusova et al., 2016). Such systems check the coherence of the prediction of specific genes, functional annotation of the genes, and cross-referencing in the databases and so flag possible errors in the annotations. Contamination Detection, Version Control and Provenance Contamination poses a severe problem in plasmid sequence databases, having possible origins in the co-purification of chromosomal DNA, sample-to-sample contamination, as well as chimeric assemblies. Profiling sequences with metagenomics tools as Kraken2 and MetaPhlAn can determine if the sequence contains DNA fragments of several bacterial species, which hints at contamination (Beghini et al., 2021). Those provide resolution on species level which makes it possible to pinpoint the source of contamination. Another approach to detecting contamination is provided by similarity of sequences through graphs, that cross-check unique detected sequences against a wider database to trace patterns of unintended similarities which suggests contaminated data or cross contamination (Ondov et al., 2016). Such methods are especially good in the case of systematic contamination of many records of sequences. Current plasmid databases apply sophisticated version control systems that track changes to data. Changes made to schemas, updated data, and other steps in the analytical pipeline are meticulously recorded on the Git-based versioning system. All steps of data processing are tracked by a sysytem and thus repetition of the process is made possible, allowing errors which have been identified to be tracked and malfunctions to be resolved. A vocabularly PROV-O enabled within the framework of a device for automated information processes outlines the domain which in turn enables representation of a data's reasoned history in a computational way. The use of blockchai, provides new opportunities for unfalsified history tracking, safeguarded by cryptographic codes that verify sets of data and analytical workflows. Visualization and User Interfaces Interactive Web Interfaces Modern plasmid databases provide sophisticated web interfaces to address diverse user needs, ranging from browsing to sophisticated analysis. Dynamic layouts are tablet, desktop, and mobile enabled. Dynamic searching includes live suggestions and filtering capabilities, enabling users to rapidly limit the number of hits. Autocomplete speeds searching from controlled vocabularies and previous searches. Phylogenetic tree viewers support zooming for examining evolutionary relationships. Network visualization tools facilitate sequence similarity and functional relationship analysis. Genome Browsers and Sequence Visualization With integrated

genome browsers, research scientists can access individual plasmid sequences and their comprehensive annotation tracks. In contemporary browsers, multiple layers of annotations, including but not limited to genes and their predictions, functional domains, regulatory constituents, and experimental data tracks, are supported. JBrowse and IGV exemplify more flexible custom genome browsers with more general use acceptance (Buels et al., 2016). These browsers enable data streaming and support plugin frameworks which allows for integration of additional specialized logical instruments. With circular topologies being the preferred structural form for plasmids, circular genome viewers are of utmost importance for plasmid visualization. Circos and other similar tools are able to create complex circular diagrams that are multilayered and allow for data comparison. Statistical Dashboards and Analytics Statistical dashboards commonly present data regarding taxonomic composition, the predominance of functional categories, and the distribution of sequence lengths. Real-time data updating capabilities allow for the production of complex statistical visualizations using interactive charting tools like D3.js and Plotly (Bostock et al., 2011); Plotly Technologies Inc., 2015. Data production rates and research trends are revealed via time series visualizations, which monitor database growth and submission trends. Patterns of research activity and sample bias are revealed by using geographic visualizations to visualize the global distribution of sequence submissions. Cloud Computing and Scalability Cloud computing platforms have successfully transformed plasmid database scalability and access through the availability of elastic computing resources and global distribution capabilities. Amazon Web Services (AWS), Google Cloud Platform (GCP), and Microsoft Azure offer end-to-end database services optimized for different patterns of workload. Amazon RDS and Google Cloud SQL provide managed relational database services with automatic backup, scaling, and maintenance capabilities. These services support quick deployment of database systems with high availability without the need for specialized database administration expertise. NoSQL cloud services such as Amazon DynamoDB and Google Firestore provide high scalability for document-oriented workloads, supporting millions of operations per second with elastic automatic response to demand patterns. Docker containerization, which offers uniform settings for development, testing, and production deployments, has emerged as the industry standard for plasmid database system deployment(Merkel, 2014). Containers remove environment-specific deployment problems by encapsulating application dependencies and configuration. The advanced container management features offered by Kubernetes orchestration platforms include fault tolerance, load balancing, and autonomous scaling (Burns & Beda, 2019). The implementation of intricate multi-component database systems with little operating overhead is made possible by these platforms. In order to improve overall system dependability through fault isolation, microservices architectures break down database systems into separate services. This allows for the independent scalability and optimization of various system components. High performance at scale is achieved by cloud-native database designs. Static content can be distributed globally through Content Delivery Networks (CDNs), which lower latency for users in other countries and increase system responsiveness in general. Multiple-level caching techniques decrease database load and enhance query performance. Application-level caching minimizes redundant calculations, whereas Redis and Memcached offer high-performance in-memory caching for frequently accessed data. Database sharding divides data among several servers according to certain standards, including taxonomic classifications or sequence properties. This method preserves query performance while allowing horizontal scaling beyond the constraints of



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This methodology outlines comprehensive and systematic approaches for the recovery and restoration of bacterial and mammalian plasmids that have been compromised by various forms of degradation, including nicks, breaks, base modifications, and structural alterations. The protocols detailed here are crucial for the assessment, purification, repair, and validation processes necessary to ensure successful plasmid restoration.
MATERIALS AND EQUIPMENTS
Table 6: DNA Isolation and Analysis Reagents
MATERIALS/ REAGENTS COMPOSITION/ SPECIFICATION SUPPLIER/ BRAND
DNA ISOLATION (MINI PREP/MIDI PREP) KIT Proprietary formulation QIAGEN, GENES2ME, SPINeasy
ISOPROPANOL Analytical Grade Sigma-Aldrich
ETHANOL 70% & Absolute Sigma-Aldrich
RNase A 20 mg/mL HiMedia
Agarose Molecular Biology Grade Figure 3: HIMEDIA (MB002-50G);Agarose
DNA Loading Dye 10X concentration Figure 4: TaKaRa; Loading Dye

DNA Ladder 1kb Ladder Figure 5: Abclonal (RM30291); DNA Ladder
Ethidium Bromide 10 mg/mL stock Sigma-Aldrich, HiMedia

Lyophilization And Blotting Materials
MCTs (1.5 ml)
Aluminium Foil
Mini Trans-Blot Accessories (BIO-RAD)
Storage Bags (Ziploc Bags)
Parafilm

Figure 6: BIO-RAD ; Trans-Blot Transfer Membranes

Table 7 : Equipment and Instrumentation
BASIC BALORATORY EQUIPMENT
EQUIPMENT MODEL / SPECIFICATIONS
Microcentrifuge Eppendorf (Centrifuge 5418 R)

Figure 7: Eppendorf 5418 R
Centrifuge Eppendorf (Centrifuge 5910 R)

Figure 8: Eppendorf 5910 R
Vortex Mixer REMI (CM 101)

Figure 9: REMI Cyclo Mixer 101
Micropipettes Thermo Scientific (P10, P20, P200, P1000)
Pipette Tips Corning
pH Meter LMPH-12

Figure 10: LMPH1 pH Meter
Analytical Balance Sartorius

Figure 11: Sartorius Practum 224-1S (220g-0.1mg) Analytical Balance
Water Bath Thermo Scientific

Figure 12: Thermo Water Bath
Heating Block BIO RAD

Figure 13 : BIO-RAD Digital Dry Bath

SPECIALIZED EQUIPMENTS
EQUIPMENT MODEL / SPECIFICATIONS
Freeze Dryer EYELA (FDU-2110)

Figure 14 : EYELA FDU-1110 Freeze Dryer
Spectrophotometer Eppendorf

Figure 15: Eppendorf BioPhotometer D30
Gel Electrophoresis System Bio-Rad

Figure 16: BIO-RAD Mini Protean Tetra System
UV Transilluminator Bio-Rad

Figure 17: BIO-RAD ChemiDoc MP
Autoclave TOMY SX-700
Laminar Flow Hood LABWIT (ZHJH-C1109C)
Incubator PRIME

STORAGE EQUIPMENT
EQUIPMENT MODEL / SPECIFICATIONS
Refrigerator (4°C ± 2°C) TRUSFROST

Figure 18:TruFrost Upright Medical Freezer,310 Ltrs
Freezer (-20°C ± 5°C) ELANPRO
Ultra-low Freezer (-80°C ± 10°C) U410 HEF

Table 8: CONSUMABLES AND SUPPLIES
ITEMS SPECIFICATION
Microcentrifuge Tubes 1.5, 2.0 mL
Falcon Tubes 15 mL, 50 mL
Culture Flasks (Conical) 250 mL, 500 mL
Disposable Gloves Nitrile, powder-free
Lab Coats Cotton/polyester blend
Safety Goggles Chemical resistant
Kimwipes Lint-free tissues
Aluminum Foil Laboratory grade
Permanent Markers Solvent resistant (Hydrophobic)
Storage Boxes Plastic, compartmented (96 wells)

Experimental Design and Statistical Framework
Sample Selection and Randomization
Stock Selection Criteria:
 Age range: 2-15 years (stored at -80°C)
 Origin: Own laboratory sources
 Vector types: Both bacterial and mammalian expression systems
 Storage conditions: Glycerol stocks in cryovials
 Replication: Each stock was processed in triplicate for statistical validation
Glycerol Stock Assessment and Revival

Initial Assessment Protocol
Visual Inspection
I. Examined stored plasmid samples for:
a. Color changes (yellowing, browning)
b. Precipitation or cloudiness
c. Container integrity
d. Storage condition documentation like Record stock age, storage temperature etc
e. Photograph each stock for documentation
II. Removed stocks from -80°C storage
III. Allowed the stocks to thaw for precisely 15 minutes, adhering strictly to standardized timing guidelines.
IV. Use a calibrated thermometer to accurately document the temperature at which thawing occurs.
V. For each stock we inoculated 5-10ml LB broth with appropriate antibiotic. We added 50 µL glycerol stock culture using sterile pipette tip heads maintaining required environment.
VI. Maintained incubation at 37°C at a rotation speed of 225 rpm for 12-16 hours.
VII. Monitored OD_{600} at 2-hour intervals (Target OD_{600}: 0.6-1.2 for optimal plasmid yield) over 16 hours.
Standardized SPINeasy Miniprep Kit Protocol (For pDNA Isolation)
i. Harvest the overnight bacterial culture (OD_{600}= 0.8-1.2) by centrifuging at 5000g for 5 mins or 10000g for 3 mins. Supernatant was removed completely (verified by weight; recorded the pellet wet weight for normalization)
ii. Resuspended the bacterial pellet in 250µLof Resuspension Buffer SN1 (RNase A added). (Cells those were in a culture tube, transferred to a microcentrifuge tube)
iii. Added 250 Alkaline Lysis Buffer SN2, mixed gently by inverting 4-6 times (No vortexing or rigorous pipetting should be employed) and incubated at room temperature (15-25°C) for 2 mins (not more than 5 mins).
Figure 19: SPINeasy DNA Plasmid Miniprep Kit
iv. Added 350 µL of prechilled Neutralization Buffer P3, mixed well by multiple invertion for 4-6times, followed by an incubation on ice for 5 min (vortexing or pipetting is restricted).
v. Centrifuged at 14000-18000g for 10 min at 4°C.
vi. Transferred all of the lysate supernatant to a Column SN with collection tube.
vii. Centrifuged at 14000g for 1 min, discarded the flow through and reused the collection tube.
viii. Added 500 µL of Wash Buffer NW1 to the column.
ix. Centrifuged at 14,000g for 1 min and discarded the flow through and again reused the collection tube.
x. Added 750 µL of Wash Buffer NW2 to the column, followed by an incubation for 1 min at room temperature.
xi. Centrifuged at 14,000g for 1 min and discarded the flow through and reutilized the collection tube.
xii. Another round of Centrifugation at 14,000g for additional 1 min is employed to dry the column completely.
xiii. Removed the collection tube and placed column into a clean 1.5 ml MCT.
xiv. Added 50 µL of Elution Buffer SN to the center of the membrane and incubated at room temperature for 10 mins.
xv. Centrifugation for one round was done at 8000g for 1-2 mins to elute the DNA.
xvi. Eluted pDNA will be collected in the associated microcentrifuge tube.
xvii. Noted any unusual colour or turbidity. Eluted pDNA was stored immediately at 4°C for analysis.

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Age range: 2-15 years (stored at -80°C)
https://www.fda.gov/files/vaccines,%2520blood%2520%26%2520biologics/published/Package-Insert-PANZYGA.pdf

Figure 19: SPINeasy DNA Plasmid Miniprep Kit

https://www.mpbio.com/media/document/file/manual/dest/s/p/i/n/e/SPINeasy_Plasmid_Miniprep_Kit.pdf%3Fsrsltid%3DAfmBOooby5Rv0KbSpUqWGjzGZK9DBv4lInzl8TE-X5cK7xVHDmXOcNOH

ix. Centrifuged at 14,000g for 1 min and discarded the flow through and again reused the collection tube.
https://www.mpbio.com/media/document/file/manual/dest/s/p/i/n/e/SPINeasy_Plasmid_Miniprep_Kit.pdf%3Fsrsltid%3DAfmBOoprHRDQd9TyN-YpYk_wSia1xzjBsRZ0dwIltT6BFSuZO9TseNWA

viii. Added 500 µL of Wash Buffer NW1 to the column.
https://www.mpbio.com/media/document/file/manual/dest/s/p/i/n/e/SPINeasy_Plasmid_Miniprep_Kit.pdf%3Fsrsltid%3DAfmBOorWT87124iGV_0WaUFURRqOcoaMzXlQY07fTcFY7-HTVUcd4Xin

xii. Another round of Centrifugation at 14,000g for additional 1 min is employed to dry the column completely.
https://www.mpbio.com/media/document/file/manual/dest/s/p/i/n/e/SPINeasy_Plasmid_Miniprep_Kit.pdf%3Fsrsltid%3DAfmBOorMM0RhbzqjLpuqMgUp9zpiMo9IaOPjhEYbo4rHVG2J-lSy5f4I

xi. Centrifuged at 14,000g for 1 min and discarded the flow through and reutilized the collection tube.
https://orbi.uliege.be/bitstream/2268/312822/1/Thesis_PhD_Anna_Caccamo_revised_final.pdf

x. Added 750 µL of Wash Buffer NW2 to the column, followed by an incubation for 1 min at room temperature.
https://www.mpbio.com/media/document/file/manual/dest/s/p/i/n/e/SPINeasy_Plasmid_Miniprep_Kit.pdf%3Fsrsltid%3DAfmBOopJsfbu465zGHvy3FVMY8MyUK09zK9ElwKRd_GQS6CD60lnXytQ

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DNA Quantification and Purity Analysis
I. Calibrated the Spectrophotometer with nuclease-free water or Elution Buffer whichever is used in mean times.
II. Measured 2 µL of each sample one by one.
III. Recorded A_{260}/A_{280} ratio, A_{260}/A_{230} ratio and absorbance.
Agarose Gel Electrophoresis Quality Assessment
I. Gel Preparation (0.8-1% Agarose)
a. Dissolved 0.4 gm / 0.5 gm in 50 mL 1X TBE/TAE Buffer
b. Heated until completely dissolved through microwave (2 minute intervals)
c. Applied 10 µL of EtBr dye, ensuring appropriate safety measures by wearing gloves during the application process.
d. Poured the whole liquid into the gel tray with 1.5 mm comb (comb size varied based on loading volumes)
e. We allowed it 30-40 minutes to polymerize at room temperature.
II. Sample Preparation and Loading
a. Mixed 3-4 µL DNA with 10X Loading Dye (1:6 ratio)
b. Loaded 10-12 µL sample-dye mixture per well.
c. Loaded samples in randomized order to prevent bias.
III. Started the run at constant 100V for approximately 45 minutes.
IV. We used 1X TBE Buffer (pH 8.0) as Running Buffer. (Subsequently transferred to the TAE Buffer for temporary storage due to time constraints)
V. The complete migration was keenly monitored, utilizing the forefront of leading loading dye with proficiency and precision.
VI. Paused/Stopped when Dye reached 80-85% of Gel Length.
VII. Image Analysis And Scoring:
a. Captured image using ChemiDoc System.
b. Used identical exposure settings (0.5 seconds).
Lyophilization Process And Cycle Development
I. Prepared lyoprotectant solutions in nuclease-free water.
II. Filter sterilization was done using 0.22 µm sterile syringe filter.
III. Stored at 4°C for short-term use
IV. Combine the purified plasmid DNA with the Lyoprotectant solution in equal volumes, maintaining a 1:1 ratio.
V. Gently pipetted to prevent DNA shearing.
VI. Aliquoted the plasmid DNA-lyoprotectant mixture into sterile, nuclease free microcentrifuge tubes which we sealed with paraffin slices with some sort of holes in it to maintain ventilation (volume

varied as we eluted the pDNA in various amounts).
VII. To standardize the drying conditions we'll separate similar volumes in a different row later.
VIII. Placed samples in a -80°C freezer overnight to freeze completely.
IX. Transferred those frozen samples to the lyophilizer (maintained vertical orientation during freezing) ; Set the vacuum to ~0.1 mbar and condenser temperature to -50°C or lower.
X. Dried for 12–24 hours, depending on sample volume and lyoprotectant composition.
XI. Increased the shelf temperature to 20–25°C for 2–4 hours to remove residual moisture, ensuring the final product forms a stable, dry cake.
XII. Sealed the lyophilized samples completely
XIII. Stored at -20°C or 4°C, depending on stability requirements.
Post-Lyophilization Quality Assessment
Table 9 : Visual Inspection Criteria in Physical Characterization Process
DNA Integrity Post-Lyophilization
I. Added exactly 50 µL nuclease-free water to lyophilized cake.
II. Incubated at room temperature for 5 minutes.
III. Carefully vortexed for a duration of 30 seconds.
IV. Permitted total dissolution (visual confirmation).
V. The analysis was conducted promptly, followed by storage at 4°C.
VI. Spectrophotometric analysis, assessing concentration and purity, alongside agarose gel electrophoresis, evaluating integrity, was conducted.
Plasmid Storage
For short-term storage (up to 6 months), purified plasmids were stored in TE buffer at -20°C.
► For long-term archiving, two complementary methods were employed:
i. Glycerol Stocks: Overnight bacterial cultures (1 ml) were mixed with sterile 50% glycerol (1 ml)(1:1 ratio) in sealed MCTs and stored at -80°C.
ii. Purified plasmid DNA (1 µg) was mixed with 10 µL of 20 mM Tris-HCl (pH 8.0) containing 3% trehalose as a cryoprotectant. Samples were lyophilized using a SpeedVac concentrator and stored at -20°C.
Blotting Paper Preservation of Bacterial and Mammalian Plasmids
Blotting Paper Preservation method relies on the adsorption of DNA onto sterile filter paper, which protects the nucleic acids from degradation while maintaining their viability for transformation experiments. The method is particularly useful for creating backup stocks of valuable plasmid constructs and for sharing plasmids between laboratories.
► Using sterile scissors, we have precisely cut the Whatman filter paper into rectangular pieces measuring x*y cm^2. Subsequently, circles were drawn on these pieces to create a disc shape for accurately dropping the DNA solution.
► The shaped papers were placed in sterile petri dishes, each containing 5 pieces of papers, and autoclaved at 121°C for 15 minutes at 15 psi. Following autoclaving, the papers were permitted to cool and dry in a sterile setting.
► Prior to utilization, all filter paper pieces were thoroughly dried to guarantee optimal condition and inspected for contamination or any signs of damage.
► All the measures and verified plasmids after the curation procedure were selected for subsequent procedures.
► Using sterile forceps, we placed individual filter paper discs on a sterile surface.
► Each circle was treated with precisely 1 µg of plasmid DNA solution applied to its center.
► Ensured even distribution without oversaturation.
► We allowed complete permeation of DNA into the paper matrix.
► The documents were permitted to dry undisturbed at stable room temperature for a period of 15 to 20 minutes.
► The drying process was conducted in a controlled, dust-free setting, ensuring full drying while carefully avoiding exposure to direct sunlight or excessive heat.
► The cut pieces were securely wrapped in aluminum foil.
► The foil-wrapped blot papers were labeled with the plasmid name, date, concentration, and the amount of DNA contained within.
► Stored at room temperature (20-25°C) under low humidity and dark environment within airtight zip logs.
Figure 20 : Schematic Overview of Blotting Paper Preservation Process

Figure 21 : DNA Recovery and Quality Control Workflow

Digital and Physical Indexing System

We have followed the systematic methodology employed for developing and implementing a physical indexing system for curated bacterial and mammalian expression vectors. This approach involves assessing collections, crafting classification frameworks, setting quality control protocols, strategizing physical organization, and integrating digital databases. Through this standardized system, we processed a total of 52 plasmid vectors.

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e. We allowed it 30-40 minutes to polymerize at room temperature.
https://www.reddit.com/r/castiron/comments/tepa4b/season_below_the_smoke_point

IV. Combine the purified plasmid DNA with the Lyoprotectant solution in equal volumes, maintaining a 1:1 ratio.
https://star-protocols.cell.com/protocols/3495

II. Incubated at room temperature for 5 minutes.
https://www.neb.com/en-us/faqs/2017/04/05/can-the-kld-reaction-be-incubated-longer-or-at-higher-temperature%3Fsrsltid%3DAfmBOoqIDOfotz74zboC4ZLJoDcVcwgXMqL7kS4N1NJezby9JSc619l7

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Consumables and Storage Materials • 1.5 mL microcentrifuge tubes (Eppendorf) • 96-well storage boxes with alphanumeric grid (Corning) • Waterproof labels Organization Hierarchy: Freezer → Rack → Box → Position → Tube Plasmid Collection Characterization Step 1: Physical Count and Documentation ► Removed all plasmid stocks from existing storage ► Conducted manual count of individual tubes ► Recorded preliminary information available on existing labels Photographed each tube for documentation ► Separated vectors by expression system based on visible markings Grouped by apparent vector series (pET, pcDNA, etc.) Figure 22: Non-Organized Plasmid Samples; 96 Well Grid Box With 49 Isolated And Purified Plasmids ► Noted any vectors requiring additional characterization. Total Plasmids (N) = Σ(Bacterial vectors) + Σ(Mammalian vectors) N = X + Y = 52 plasmids Step 2: Categorization Calculations: ► Bacterial vector percentage = (x/52) × 100 ► Mammalian vector percentage = (y/52) × 100 Figure 23 : Distribution of Bacterial and Mammalian Plasmids; Bar graph showing the number of bacterial and mammalian plasmids Step 3: Detailed Vector Series Identification And Classification An identification protocol was systematically applied to each plasmid: ► Examined existing labels for vector name and series information ► When necessary, performed restriction digest analysis (We performed UBCH10) Distribution calculation methodology: For each vector series i: Percentage of series, i = (Count of series i / Total plasmids) × 100 Step 5: Nomenclature Standardization Standardized Naming Convention Development: [System Prefix]-[Sequential Number]: [Original Name] Where: - BAC: Bacterial expression system - MAM: Mammalian expression system - Sequential number: 001-052 (zero-padded) ► Assigned system prefix based on classification ► Numbered sequentially within each category ► Maintained the original name for reference Step 6: Physical Organization Protocol And Storage Infrastructure Setup ► Primary Storage: -20°C freezer ► Container: 2 × 96-well storage boxes ► Capacity Utilization: 52/192 positions = 27.08 % ► Reserved Expansion: 140 positions available Box Organization Strategy: ► Box 1: BAC-001 to BAC-017 + MAM-001 to MAM-009 ► Box 2: MAM-010 to MAM-035 + Reserved positions TABLE 10 : Systematic Classification of Plasmid Stocks INDEX PLASMID NAME CATEGORY VECTOR SERIES NOTES FOR FUTURE RESTORATION/ VALIDATION BAC-001 pGEX-4T1 BACTERIAL pGEX Verify supercoiled DNA via gel; test transformation efficacy BAC-002 TA-4 BACTERIAL TA Confirm insert via PCR; test cloning efficiency. BAC-003 pGEX-GM3 #3 BACTERIAL pGEX Quantify supercoiled DNA via ImageJ; test protein expression. BAC-004 TA-3 BACTERIAL TA Verify insert integrity. BAC-005 TA-361BP BACTERIAL TA Confirm insert size via PCR and gel electrophoresis. BAC-006 PGEX-5X-2-GM4 BACTERIAL pGEX Quantify supercoiled DNA; test GST-tagged protein expression. BAC-007 UBCH10 BACTERIAL pET Test Protein Expression in BL21(DE3) cells BAC-008 U61 BACTERIAL Unique/Ambiguous Confirm cloning efficiency; verify insert. BAC-009 PG5E4 IN DH5α BACTERIAL pGEX Confirm insert via PCR; test transformation efficiency. BAC-010 PBS-EMP BACTERIAL pBS Use as cloning control; verify insert absence. BAC-011 TA-1 BACTERIAL TA Verify

insert integrity. BAC-012 TA-2 BACTERIAL TA Verify insert integrity. BAC-013 PSD 4 BACTERIAL Unique/Ambiguous Confirm cloning efficiency; verify insert. BAC-014 PJS55-CDC20 BACTERIAL Unique/Ambiguous Test CDC20 expression; sequence for integrity BAC-015 ΔN UBCH10 BACTERIAL pET Test UBCH10 expression; sequence deletion region. BAC-016 pGEX-5X-2 BACTERIAL pGEX Verify GST tag functionality; confirm tac promoter activity in E. coli BL21(DE3); sequence insert for integrity. BAC-017 ΔC pGEX-5X-2 BACTERIAL pGEX Check impact of C-terminal deletion on GST fusion protein stability; test expression levels in E. coli BL21(DE3); sequence to confirm deletion boundaries. MAM-001 AUF1-sh(hNRPD-sh LENTI) MAMMALIAN Lentiviral Test transfection efficiency in HEK293 cells; ensure low endotoxin MAM-002 CUEDC2-S110A MAMMALIAN pCMV/pcDNA Validate expression in mammalian cells; sequence for S110A mutation MAM-003 CUEDC2-WT MAMMALIAN pCMV/pcDNA Compare expression with S110A mutant; verify sequence MAM-004 pGL3 181 BP E2 PROMOTER MAMMALIAN pGL3 Compare luciferase activity with 733 bp variant MAM-005 pGL3 733 BP E2 PROMOTER MAMMALIAN pGL3 Test luciferase activity in mammalian cells; quantify promoter strength MAM-006 CDC20 pTRE-TIGHT MAMMALIAN pTRE-Tight Test Tet-On/Off inducibility; verify CDC20 expression MAM-007 H3-CMV-pFLAG MAMMALIAN pTRE-Tight Validate FLAG-tagged protein expression via Western Blot MAM-008 LYS MUT CDC20 MAMMALIAN pCMV/pcDNA Sequence for lysine mutation; test cell cycle effects MAM-009 MAD1-GFP MAMMALIAN pEGFP Assess GFP fluorescence in mammalian cells MAM-010 ΔN- PEGFP MAMMALIAN pEGFP Validate GFP expression; sequence deletion region MAM-011 ΔC-PEGFP MAMMALIAN pEGFP Check expression levels MAM-012 pEGFP-N2 EMPTY VECTOR MAMMALIAN pEGFP Use as a control for pEGFP-based plasmids; test transfection MAM-013 PGL3-361 BP E2 PROMOTER MAMMALIAN pGL3 Test luciferase activity; compare with other pGL3 variants MAM-014 PGL3-983 BP MAMMALIAN pGL3 Similar to PGL3-361BP; assess promoter strength MAM-015 R4-PEGFP MAMMALIAN pEGFP Validate GFP expression; confirm insert presence MAM-016 RFP-MAD2 MAMMALIAN pRFP Assess RFP fluorescence in mammalian cells MAM-017 PTRE-TIGHT EMPTY VECTOR MAMMALIAN pTRE-Tight Use as control for pTRE-TIGHT plasmids; test inducibility MAM-018 pcDNA-NUP MAMMALIAN pcDNA Validate NUP expression; ensure low endotoxin levels MAM-019 APC-5 MAMMALIAN pCMV/pcDNA Test expression in mammalian cells; verify sequence MAM-020 E2F1-MUT 361BP UBCH10-PGL3 MAMMALIAN pGL3 Test luciferase activity; compare with wild-type promoter MAM-021 PTRE-TIGHT LUCIFERASE MAMMALIAN pTRE-Tight Assess the inducibility of the pTRE-Tight Test luciferase in mammalian cell systems. MAM-022 MAD1-3`-UTR MUTATION MAMMALIAN pGL3 Assess regulatory effects in mammalian cells MAM-023 pCMV-MYC-NUP MAMMALIAN pCMV Validate MYC-tagged protein expression MAM-024 PEGFP-CDC20 MAMMALIAN pEGFP Assess GFP fluorescence; validate CDC20 function MAM-025 PGL3-REV-E2-983BP MAMMALIAN pGL3 Test luciferase activity; compare with forward promoter MAM-026 PEDZ-6 MAMMALIAN Unique/Ambiguous Test transfection efficiency; ensure low endotoxin. MAM-027 PGL3 BASIC MAMMALIAN pGL3 Use as cloning control; verify insert absence. MAM-028 N3-CDC20-GFP MAMMALIAN pEGFP Assess GFP fluorescence; validate CDC20 function MAM-029 PEFGP-GM3 MAMMALIAN pEGFP Validate GFP expression; confirm insert MAM-030 pcDNA3.1 MAMMALIAN pcDNA Protein Expression MAM-031 APC 7 MAMMALIAN pCMV/pcDNA Protein Expression MAM-032 NLS-MUT-FL-CDC20-PEGFP MAMMALIAN pEGFP Fluorescent Tagging validation needed. MAM-033 BAC-A9 (PEB-9) MAMMALIAN PEB-9 Test stability in DH10B cells; verify insert size. BACV-001 PACHLT-A EMPTY BACULOVIRUS TRANSFER VECTOR; MAMMALIAN Baculovirus Transfer (pAcHLT) Confirm the integrity of the polyhedrin promoter and MCS by sequencing, as baculovirus transfer vectors may degrade over time, affecting recombination efficiency. BACV-021 PACHLT-A-CDC20 BACULOVIRUS TRANSFER VECTOR; MAMMALIAN Baculovirus Transfer (pAcHLT) Validate CDC20 expression; test cell cycle effects. Labeling System Implementation: ► Primary Labels: Waterproof labels with index code ► Color Coding: • Blue dots: Bacterial vectors (n= 17) • Red dots:

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freezer ► Container: 2 × 96-well storage boxes ► Capacity Utilization: 52/192 positions = 27.08 % ► Reserved Expansion: 140 positions available Box Organization Strategy: ► Box 1: BAC-001 to

https://www.diaago.com/shop/racks/freezer-rack-for-0-2-ml-tube-96-well-pcr-boxes

MAMMALIAN pEGFP Validate GFP expression; sequence deletion region MAM-011 ΔC-PEGFP MAMMALIAN pEGFP Check expression levels MAM-012 pEGFP-N2 EMPTY VECTOR MAMMALIAN pEGFP Use as a control for pEGFP-based plasmids; test transfection

https://www.realgenelabs.com/pegfp-n2-mammalian-expression-vector-514014

pCMV/pcDNA Protein Expression MAM-032 NLS-MUT-FL-CDC20-PEGFP MAMMALIAN pEGFP Fluorescent Tagging validation needed.

https://www.thermofisher.com/order/catalog/product/V79020

Nomenclature Standardization Standardized Naming Convention Development: [System Prefix]-[Sequential Number]: [Original Name] Where: - BAC: Bacterial expression system - MAM: Mammalian expression system - Sequential number: 001-052 (zero-padded) ▶ Assigned system

https://www.bioprocessonline.com/doc/mammalian-expression-system-0001

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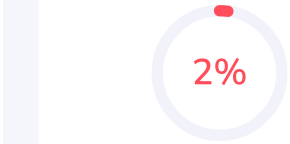

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Step 7: Quality Control Assessment Protocol
Sample Preparation:
► Thawed plasmid stocks on ice (10 minutes)
► Mixed gently by pipetting
► Aliquoted 2 µL for analysis
► Returned the sample to the stock to store immediately
► Concentration Measurement:
► Method: NanoDrop spectrophotometry (wavelength- 260nm) (A260/A280: 1.8-2.0)
► Blank: TE buffer or nuclease free water.
► Volume required: 2-5 µL per sample (DNA Conc- ≥100 ng/µL)
Figure 24 : Spectrophotometric analysis

Integrity Verification:
► Performed 0.8 % agarose gel electrophoresis with Ethidium Bromide staining and 1kb DNA ladder standard.
► Samples showing degradation or unusual migration patterns were excluded from analysis.
► Inspected under ChemiDoc UV Illuminator and analyzed.

Figure 25: Gel Inspection and analysis under ChemiDoc. Figure 26 : BIO-RAD ChemiDoc

Step 8: Digital Database Integration
Database Schema
♦ Index ID (Primary Key)
♦ Plasmid Name
♦ Vector Backbone
♦ Backbone Size
♦ Expression system

♦ Promoter
♦ Selection marker
♦ Concentration (ng/µL)
♦ Storage location
♦ Preparation date
♦ Date Indexed
♦ Date Of Last Use
♦ Quality control status (Descriptive Band Pattern Notation, Pattern With Intesity, Band Pattern Type)

Step 9: Data Entry and Validation

► Entered basic pieces of information (name, category, series)
► Added quality control measurements to the Spreadsheet (Excel, WPS Office)
► Assigned the proper storage location
► Validated and cross-checked all entries for completeness
► Created one backup database

Table 11 : Step 10: Maintenance Protocol

WEEKLY TASKS MONTHLY TASKS
 Verified storage temperatures
 Checked label integrity
 Update usage logs Quality spot-checks (random sampling)
 Database backup
 Inventory reconciliation

Step 11: Quality Control Assessment

To validate the integrity, we first performed Single Enzyme Digestion to see if the digestion is occurring or not. We used UBCH10 (hUBCH10-PET29) for assessment.

► Plasmid Details: hUBCH10-pET29 construct (~6.2 kb total, with 459 bp hUBCH10 insert)
► Antibiotic Selection: Kanamycin resistance (50 µg/mL) for pET29 vector

Figure 27: Plasmid Map of pET29a+ vector; taken from SnapGene (Dotmatics)

Figure 28: Plasmid Map of the hUBCH10-pET29 construct. Plasmid has a total size of 6.2kb, including a 450 bp insert (hUBCH10). Key features include kanamycin resistance (KanR, 50 µg/mL) for selection in pET Vector, along with annotated restriction sites and functional elements (Biosoft)

► hUBCH10-pET29 plasmid DNA was isolated using GENES2ME SPINXT Plasmid DNA Extraction Kit.
► DNA concentration was measured using BioRad spectrophotometer.
► Purity was assessed by A260/A280 ratio (acceptable range: 1.8-2.0)
► Expected plasmid size: ~ 6.2 kb (hUBCH10 insert ~459 bp + pET29 vector ~5.7 kb)
► Agarose gel electrophoresis (0.8% gel) was performed to assess plasmid integrity

Figure 29 : GENES2ME SPINXT Plasmid Miniprep Kit

Single Enzyme Digestion with XhoI

1. Reaction Setup (50 µL total volume):
 Plasmid DNA: 1 µg
 XhoI Restriction enzyme: 1 unit per µg of DNA
 CutSmart Buffer (10×): 5 µL
 Sterile water: to final volume of 50 µL
2. Digestion Conditions:
 Incubation temperature: 37°C
 Incubation time: 1-2 hours
 Heat inactivation: 65°C for 20 minutes (optional)
3. Expected Results:

► XhoI cuts at single site in pET29a+ multiple cloning site

► Expected linearized plasmid: ~6.2 kb

Figure 30: Restriction Digestion Setup; Setting Water Bath temperature to 37 degree C

Double Enzyme Digestion with EcoRI and XhoI

Reaction Setup (50 µL total volume):

 Plasmid DNA: 1 µg

 XhoI Restriction enzyme: 1 unit per µg of DNA

 EcoRI Restriction enzyme: 1 unit per µg of DNA

 TaKaRa Buffer (10×): 5 µL

 Sterile water: to final volume of 50 µL

Figure 31: Restriction Buffer (TaKaRa)

Figure 32: Restriction Enzymes- XhoI & EcoRI (TaKaRa)

Expected Results:

► EcoRI cuts upstream of hUBCH10 insert

► XhoI cuts downstream of hUBCH10 insert

► Expected fragments:

i. hUBCH10 insert: ~459 bp

ii. pET29 vector backbone: ~5.7 kb

Gel Electrophoresis and Analysis

► We prepared 1.5 % agarose gel with 1× TAE buffer.

► We added EtBr for visualization

► We used 1kb molecular weight markers.

► Electrophoresis Conditions:

a. Voltage: 100V

b. Running time: 45-60 minutes

c. Buffer: 1× TAE

Figure 33: 1kb DNA Ladder (RM30291)

RESULTS AND DISCUSSION

An evaluation was conducted on the effectiveness of different storage methods for plasmid restoration in order to enhance the protocols for managing the plasmid repository. A comprehensive restoration process was executed on 16 bacterial and 36 mammalian expression plasmids, sourced from both glycerol stocks and lyophilized preparations, which had been preserved for a period ranging from 2-15 years.

Plasmid Inventory and Classification

Table 12 : Percentage Distribution of Bacterial and Mammalian Plasmids

CATEGORY NUMBER OF PLASMIDS PERCENTAGE(%)

BACTERIAL 17 32.69

MAMMALIAN 35 67.30

Table 13 : Summary of Plasmid Collection

PLASMID TYPE (CLONED GENE NAME) NUMBER OF SAMPLES EXPRESSION SYSTEM STORAGE CONDITION

pGEX Series 6 tac promoter -80°C glycerol stocks

pET Series 2 T7 promoter -80°C glycerol stocks

pEGFP Series 8 CMV -80°C glycerol stocks

pTRE-TIGHT Series 3 TRE-Tight -80°C glycerol stocks

TA Series 5 N/A (lac for resistance) -80°C glycerol stocks

pCMV/pcDNA Series 10 CMV -80°C glycerol stocks

PBS Series 1 N/A (lac for resistance) -80°C glycerol stocks

BAC Series 1 N/A -80°C glycerol stocks

Lentiviral Series 1 U6 (for shRNA) -80°C glycerol stocks

pGL3 Series 8 E2 (PGL3 BASIC: None) -80°C glycerol stocks

pFLAG, pRFP Series 2 CMV -80°C glycerol stocks

Unique or ambiguous vectors (Bacterial & Mammalian) 4 Unknown (likely lac or T7), Unknown (likely CMV) -80°C glycerol stocks

Percentage Distribution of Plasmid Series

Figure 34 : This Bar graph visualizes the percentage of plasmids in each series (e.g., pGEX, pGL3), as calculated previously: (pGEX: 11.54% , TA: 9.62% ,pET: 3.85% ,Unknown (Bacterial): 5.77% ,pBS: 1.92% ,BAC: 1.92% ,Lentiviral: 1.92% ,pcDNA/pCMV: 19.23% ,pGL3: 15.38%, pTRE-Tight: 5.77% ,pFLAG: 1.92% ,pEGFP: 15.38% ,pRFP: 1.92% ,Unknown (Mammalian): 1.92%)

Plasmid DNA Yield and Purity Assessment
Plasmid DNA was extracted using the standard alkaline lysis method. Yield and purity were assessed spectrophotometrically.

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□ EcoRI Restriction enzyme: 1 unit per µg of DNA
https://www.neb.com/en-us/products/r0101-ecori%3Fsrsltid%3DAfmBOorgkR8CTReRnF50YJJJiNPUP8Tk9fxPtsRLEvKMvt_Gez1K6IOT

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► Blank: TE buffer or nuclease free water.
https://www.reddit.com/r/labrats/comments/196tvha/nuclease_free_te_buffer

► Thawed plasmid stocks on ice (10 minutes)
https://www.neb.com/en-us/tools-and-resources/usage-guidelines/chemical-transformation-tips%3Fsrsltid%3DAfmBOopmAN3gFRcMfXhnLAO6dv5hTHcMvSNPDQb-jagn_atiVOHVgQIl

► Antibiotic Selection: Kanamycin resistance (50 µg/mL) for pET29 vector
https://www.addgene.org/49788

□ Sterile water: to final volume of 50 µL
https://pmc.ncbi.nlm.nih.gov/articles/PMC7754154

□ Sterile water: to final volume of 50 µL
https://pmc.ncbi.nlm.nih.gov/articles/PMC7754154

□ Heat inactivation: 65°C for 20 minutes (optional)
https://www.neb.com/en-us/tools-and-resources/usage-guidelines/heat-inactivation

► hUBCH10-pET29 plasmid DNA was isolated using GENES2ME SPINXT Plasmid DNA Extraction Kit.
https://www.genes2me.com/brochure/G2M%2520Extraction%2520Solutions.pdf

► We prepared 1.5 % agarose gel with 1× TAE buffer.

https://www2.nau.edu/~fpm/Lab%2520Protocols/Agarose_GelElectrophoresis.pdf

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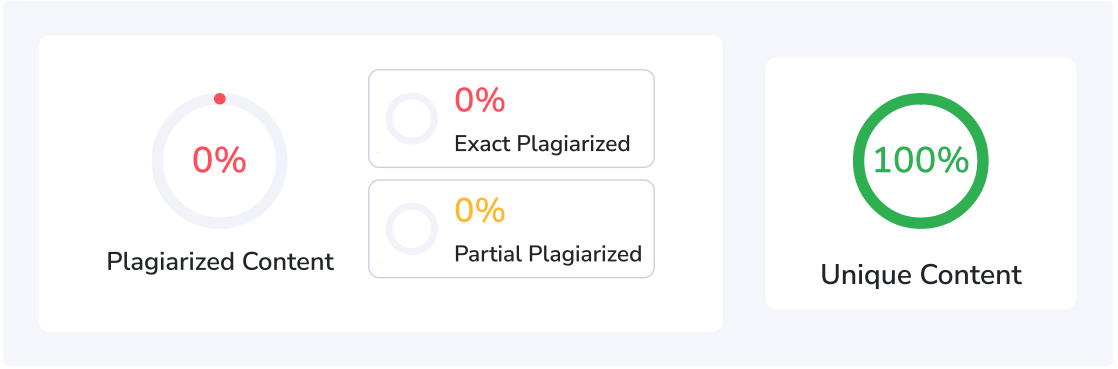

Total Words: 861 **Total Characters:** 5809 **Plagiarized Sentences:** 0 **Unique Sentences:** 18 (100%)

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Table 14 : Plasmid DNA Extraction Results

SAMPLE/ INDEX ID PLASMID NAME DNA CONC. (ng/µL) A260/A280 A260/A230 Total Yield (µg)
BAC-001 pGEX-4T1 406.9 1.83 2.29 16.27
BAC-002 TA-4 202.7 1.85 2.49 8.108
BAC-003 pGEX-GM3 #3 737.4 1.87 2.23 29.49
BAC-004 TA-3 686.7 1.93 2.18 27.46
BAC-005 TA-361BP 224.3 1.80 1.74 6.72
BAC-006 PGEX-5X-2-GM4 412.3 1.78 1.46 16.49
BAC-007 UBCH10 365.5 1.89 2.44 14.62
BAC-008 U61 1463.1 1.78 2.01 58.524
BAC-009 PG5E4 IN DH5α 1445.8 1.79 2.24 57.93
BAC-010 PBS-EMP 473.4 1.84 2.40 23.67
BAC-011 TA-1 512.9 1.95 2.55 20.51
BAC-012 TA-2 1202.0 1.95 2.54 48.08
BAC-013 PSD4 103.9 1.84 2.37 3.11
BAC-014 PJS55-CDC20 1463.1 1.81 2.19 190.20
BAC-015 ΔN UBCH10 104.3 1.79 2.37 3.12
BAC-016 pGEX-5X-2 1311.2 1.99 2.56 91.78
BAC-017 ΔC pGEX-5X-2 1446.8 2.02 2.49 144.68
MAM-001 AUF1-sh(hNRPD-sh LENTI) 293.4 1.78 2.21 14.67
MAM-002 CUEDC2-S110A 309.9 1.97 2.43 12.39
MAM-003 CUEDC2-WT 1195.6 1.91 2.28 83.69
MAM-004 pGL3 181 BP E2 PROMOTER 202.7 2.04 2.38 8.10
MAM-005 pGL3 733 BP E2 PROMOTER 269.3 2.00 2.27 13.46
MAM-006 CDC20 pTRE-TIGHT 397.5 1.93 2.29 15.90
MAM-007 H3-CMV-pFLAG 695.8 1.89 2.29 27.83
MAM-008 LYS MUT CDC20 502.2 1.97 2.36 20.08
MAM-009 MAD1-GFP 497.7 1.80 1.47 19.90
MAM-010 ΔN-PEGFP 1077.2 1.95 1.58 75.40
MAM-011 ΔC-PEGFP 276.4 1.79 2.03 8.29
MAM-012 pEGFP-N2 EMPTY VECTOR 173.4 1.75 1.36 5.20
MAM-013 PGL3-361 BP
E2 PROMOTER 223.3 1.84 1.75 6.69
MAM-014 PGL3-983BP
E2 PROMOTER 412.3 1.76 1.48 16.49

MAM-015 R4-PEGFP 80.5 1.85 1.74 3.22

MAM-016 RFP-MAD2 237.1 1.86 1.78 7.11
MAM-017 PTRE-TIGHT EMPTY VECTOR 88.3 1.79 1.65 3.53
MAM-018 pcDNA-NUP 788.0 1.88 2.16 31.52
MAM-019 APC-5 949.3 1.93 2.46 66.45
MAM-020 E2F1-MUT 361BP UBCH10-PGL3 1121.2 1.89 2.44 44.84
MAM-021 PTRE-TIGHT LUCIFERASE 725.5 2.08 2.09 29.02
MAM-022 MAD1-3`-UTR MUTATION 1384.4 1.89 2.50 55.37
MAM-023 pCMV-MYC-NUP 1247.2 1.97 2.56 49.88
MAM-024 pEGFP-CDC20 1272.1 2.04 2.52 87.30
MAM-025 PGL3-REV-E2-983BP 1269.1 1.97 2.45 50.76
MAM-026 PEDZ-6 397.7 1.98 2.34 11.93
MAM-027 PGL3 BASIC 1041.8 1.88 2.28 41.67
MAM-028 N3-CDC20-GFP 564.3 1.92 2.50 16.92
MAM-029 PEFGP-GM3 641.8 1.96 2.44 25.67
MAM-030 pcDNA3.1 930.0 1.88 2.34 37.20
MAM-031 APC 7 157.0 1.79 2.41 4.71
MAM-032 BAC-A9 (PEB-9) 1329.5 1.75 1.75 53.18
MAM-033 NLS MUT FL CDC20 PEGFP 372.7 1.85 2.11 11.18
MAM-033/ BACV-001 PACHLT-A EMPTY 734.0 1.82 2.31 29.36
MAM-034/BACV-002 PACHLT-A-CDC20 1232.4 1.95 2.51 49.29
-
Figure 35 : DNA Concentration Distribution Bar Chart showing distribution of DNA concentrations across all samples, X-axis: DNA concentration ranges (ng/μL) and Y-axis: Number of samples in each concentration range; The distribution shows that most plasmid samples have concentrations between 200-800 ng/μL, with a smaller group of high-concentration samples (1200-1500 ng/μL)]
► Total samples: 52
► Mean concentration: 734.5 ng/μL
► Median concentration: 489.9 ng/μL
► Range: 80.5 - 1463.1 ng/Ml
Key Observations:
1. The bimodal distribution is distinctly observable, characterized by two separate peaks.
2. Primary cluster: ~33 samples between 80-800 ng/μL (lower concentration group)
3. Secondary cluster: ~12 samples between 1000-1500 ng/μL (high-yield group)
4. Gap between clusters: Very few samples in the 800-1000 ng/μL range
Sample Quality Assessment:
• Excellent yields (>1000 ng/μL): 12 samples (23%)
• Good yields (400-1000 ng/μL): 18 samples (35%)
• Moderate yields (200-400 ng/μL): 16 samples (31%)
• Low yields (<200 ng/μL): 6 samples (11%)

Figure 36 : Purity Assessment ; scatter plot with A260/A280 ratios on X-axis and A260/A230 ratios on Y-axis. Scatter plot of A260/A280 vs. A260/A230 ratios for 52 plasmids. Green dashed lines indicate the acceptable A260/A280 range (1.8–2.0), and red dashed lines indicate the acceptable A260/A230 range (2.0–2.2). Outliers (e.g., pEGFP-N2 EMPTY VECTOR, PTRE-TIGHT LUCIFERASE) are annotated.
Agarose Gel Electrophoresis Analysis

All isolated plasmids were subjected to agarose gel electrophoresis (0.8-1% agarose in TAE buffer) to verify structural integrity.
FIGURE 37: Agarose gel electrophoresis confirming structural integrity of isolated plasmids MAM-033 and BAC-001, both displaying characteristic double band patterns.

 MAM-033
DOUBLE BAND PATTERN: Two bands of similar intensity in upper half of gel

 BAC-001
(DOUBLE BAND PATTERN: Two bands of similar intensity in upper half of gel (Doublet /Twin Bands), shortly separated)

FIGURE 38: Gel electrophoresis analysis of MAM-001 plasmid displaying characteristic single band in upper gel region

 MAM-001
(SINGLE BAND: One sharp band in upper half of gel)

FIGURE 39: Agarose gel electrophoresis of plasmid MAM-003 showing double band pattern with widely separated bands of similar intensity.

 MAM-003
(DOUBLE BAND PATTERN: Two similar band of same intensity and width in upper half of gel, widely separated)

FIGURE 40: Gel electrophoresis analysis of BAC-003 displaying complex band pattern with one high intensity band and two lower intensity bands

 BAC-003
(COMPLEX BAND PATTERN: One high intensity band with other two low intensity bands, shortly separated, sort of smears are visible, RNA contamination is present)

FIGURE 41: Comparative gel electrophoresis of plasmids MAM-005, BAC-007, and MAM-004 showing distinct double band patterns for each construct.

 MAM-005
(DOUBLE BAND PATTERN: Two bands of similar intensity in upper half of gel, shortly separated, RNA Contamination at hand)

 BAC-007
(DOUBLE BAND PATTERN: Two bands of different intensities in upper half of gel; one comparatively more intense than the other, widely separated)

 MAM-004
(DOUBLE BAND PATTERN: More or less of similar intensity in the upper half of gel, strictly separated, sort of RNA contamination is there)

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MAM-005 pGL3 733 BP E2 PROMOTER 269.3 2.00 2.27 13.46
https://mikomarine.com/underwater-magnets/mam-005

MAM-017 PTRE-TIGHT EMPTY VECTOR 88.3 1.79 1.65 3.53
https://www.snapgene.com/plasmids/mammalian_expression_vectors/pTRE-Tight

MAM-012 pEGFP-N2 EMPTY VECTOR 173.4 1.75 1.36 5.20

https://www.snapgene.com/plasmids/fluorescent_protein_genes_and_plasmids/pEGFP-N2

MAM-033/ BACV-001 PACHLT-A EMPTY 734.0 1.82 2.31 29.36

https://www.snapgene.com/plasmids/insect_cell_vectors/pAcHLT_A

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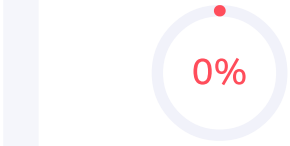

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FIGURE 42: Gel electrophoresis of plasmids MAM-006, MAM-007, and MAM-008 demonstrating varied banding patterns from complex to double band configurations

 MAM-006
(COMPLEX PATTERN: Faint smeared high MW band with two distinct lower bands)

 MAM-007
(COMPLEX PATTERN: Proper One high MW band with other three distinct lower MW bands)

 MAM-008
(DOUBLE BAND PATTERN: Two bands of similar intensity, precisely separated)

FIGURE 43: Agarose gel electrophoresis of BAC-004 and BAC-013 showing double and single band patterns with different molecular weight distributions.

 BAC-004
(DOUBLE BAND PATTERN: Two bands of different intensity and molecular weights respectively, high MW band is following the latter)

 BAC- 013
Single Band Pattern: One faint upside low density band has appeared

FIGURE 44: Agarose gel electrophoresis showing DNA band patterns for samples BAC-005, MAM-010, BAC-013, MAM-011, BAC-006, MAM-012, MAM-013, and MAM-014. Multiple discrete bands of varying molecular weights are visible across lanes.

 BAC-005
(TRIPPLE BAND PATTERN: High MW band is in the last with two lower MW bands leading, smear is

noticeable)

 MAM-010
SINGLE BAND: One distinct band (low width) is evident (~top 1/4 of gel)

 BAC-012
(Thin one band is apparent)

 MAM-011
(Smeared appeareance along the lane)

 BAC-006
SINGLE BAND: Neat band (high MW) is evident (~top 1/4 of gel)

 MAM-012
SINGLE BAND: Neat band (high MW) is apparent (~top 1/4 of gel)

 MAM-013
DOUBLE BAND PATTERN: One clear band of high MW is prominent with one partly segregated low MW band (noticeable)

 MAM-014
DOUBLE BAND PATTERN: Neat band of high MW is prominent with one partly pulled low MW band

FIGURE 45: Gel electrophoresis results displaying samples MAM-015 (double band pattern with higher and lower intensity bands), MAM-016 (double band pattern with different intensities), and MAM-017 (single sharp band in mid-lane position).

 MAM-015
DOUBLE BAND PATTERN: One band with higher intensity with a lower intensity higher MW band

 MAM-016
DOUBLE BAND PATTERN: Two different bands with different intensities

 MAM-017
SINGLE BAND: One sharp band evident in the mid-way of lane

FIGURE 46: Six-lane electrophoresis gel displaying samples BAC-007 (double band pattern), MAM-018 (single elevated band), MAM-019 (double band pattern with nearly similar bands), MAM-020 (double band pattern with indifferent intensities), MAM-031 (empty lane), and MAM-034/BACV-002 (single high-intensity band with evident contamination)

 BAC-007
DOUBLE BAND PATTERN: Two bands of similar intensity, ill-defined

 MAM-018
SINGLE BAND apparent with elevated acuteness(high MW)

 MAM-019
DOUBLE BAND PATTERN: Two nearly similar bands in size with minimal different intensities are prominent, separation is straightforward

 MAM-020
DOUBLE BAND PATTERN: Two nearly similar bands in size with quite indifferent intensities are prominent, broadly separated

 MAM-031
Lane is empty

 MAM-034/BACV-002
Single High Intensity Band with evident MW, separation is very lucid, no contamination is anticipated so far

FIGURE 47: Electrophoresis gel showing sample MAM-021 with two discrete bands of modest intensity, demonstrating tangible band transparency.

 MAM-021
Two discrete bands with modest intensity, transparency is tangible

FIGURE 48: Multi-lane gel analysis showing samples MAM-022 (triple band pattern with fragile, modest intensity bands), BAC-008 (two similar bands with analogous intensities), and MAM-032 (two feeble bands with obtained smears).

 MAM-022
TRIPPLE BAND PATTERN: Relatively fragile, modest intensity and band strength is little compromised in middle band , smudges are emerging

 BAC-008
TWO SIMILAR BANDS with analogously similar band width and intensity, separation is exact, smears are marked

 MAM-032
Two feeble bands(same MW) are obtained, smears are intense

FIGURE 49: Lane analysis reveals BAC-012 with triple band pattern indicating high MW bands, MAM-001 displaying single band pattern, MAM-023 showing empty lane, and BAC-014 exhibiting double band pattern with discrete high MW bands.

 BAC-012
TRIPLE BAND PATTERN: One intense moderate MW band(~top 1/4 of gel), two other with nearly similar width, unequally distributed bands are residing underneath, smears are evident

 MAM-001
Single Band evident

 MAM-023
Lane is empty

 BAC-014
DOUBLE BAND PATTERN: Two discrete fierce (high MW) bands are detectable with massive smear content

FIGURE 50: Electrophoretic analysis of restored plasmids demonstrating varied banding patterns. MAM-024 shows double band pattern with different sized bands, MAM-025 exhibits complex smearing pattern with indistinct bands, and MAM-033 displays double bands of different molecular weights.

 MAM-024
DOUBLE BAND PATTERN: Two different bands(one is high MW,other is lower MW), distribution is proper, smears are somewhat extant

 MAM-025
Extensive smeared lane, COMPLEX PATTERN: Thick intense band is leading, other two most indistinct bands remaining in the middle and last moderate one runnning late

 MAM-033
DOUBLE BAND: Bands are muzzy and are of different sizes (MW)

FIGURE 51: Multiple samples (MAM-031, MAM-009, BAC-015, MAM-033/BACV-001, MAM-011, MAM-026, BAC-010) demonstrate varying band intensities and patterns, indicating different levels of DNA recovery and integrity.

 MAM-031
Degraded DNA along the lane, intensity is also compromised
 MAM-009
TRIPPLE BAND PATTERN: High MW band is in the last with two lower MW bands leading, smear is instant
 BAC-015
Slight Single Faint band is visible, huge smear seizure
 MAM-033/BACV-001
DOUBLE BAND PATTERN: ONE BAND IS OF HIGHER INTENSITY, Integrity is endangered
 MAM-011
COMPLEX BAND PATTERN: Three feeble bands clearly evident, least distinct one is running after the leader band(similarly alike)
 MAM-026
COMPLEX PATTERN: Smeared high MW band with three imperceptible bands of lower MWs
 BAC-010
COMPLEX PATTERN: Smeared high MW band (crisp) with three nearly obscure bands of lower MWs

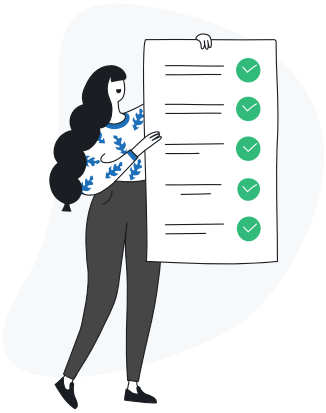

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 FIGURE 52: Samples MAM-027 through MAM-023 exhibit varied electrophoretic profiles including single low MW bands, discrete separated bands, thick intense bands, and complex smearing patterns, demonstrating successful DNA recovery with different molecular weight distributions.

 MAM- 027
Very obscure SINGLE LOW MW BAND with significant smears along the lane, smears are apparent
 MAM-028
Two discrete, fatigued non-similar(MW) bands, smears are proper, widely spread
 BAC-011
Three distinct (almost similar MW) vague bands, separated clearly(one at a distance, other twos are aligning nearby)
 BAC-012
Thin one band is apparent
 MAM-029
DOUBLE BOND PATTERN: One thick intense band & other is too obscure relatively with lower MW, smears are clear
 MAM-025
COMPLEX PATTERN: Thick intense band is leading, other two most indistinct bands remaining in the middle and last moderate one runnning late
 BAC-016
Smeared pattern, no distinct band visible
 MAM-023
No bands were visible, lane is smeared

 FIGURE 53: BAC-004 displays single band pattern with moderate intensity and visible smearing, BAC-017 exhibits triple band pattern with one intact high MW band and two partially separated bands of different intensities, and MAM-010 shows two distinct bands with similar intensity and available smears.

 BAC-004
Single Band pattern, moderate intensity, Smears are present

 BAC-017
TRIPPLE BAND PATTERN: One intact low MW band is ahead of two partially separated bands of

different intensities.

 MAM-010
Two distinct bands with similar intensity, smears available

Table 15 : Gel Electrophoresis Results Summary

SAMPLE ID BAND QUALITY BAND INTENSITY (1= FAINT, 5= INTENSE) INTEGRITY STATUS
BAC-001 pGEX-4T1 HIGH MW- 5,MEDIUM MW- 4 GOOD
BAC-002 TA-4 HIGH MW- 2, LOW MW- 4 GOOD
BAC-003 pGEX-GM3 #3 HIGH MW- 3, LOWER MW- 1,1 MODERATE
BAC-004 TA-3 HIGH MW- 5, LOW MW- 4 GOOD
BAC-005 TA-361BP HIGH MW- 4, LOWER MWs- 3,3 GOOD
BAC-006 PGEX-5X-2-GM4 SINGLE UP-RIGHT POSITIONED BAND- 5 GOOD
BAC-007 UBCH10 HIGH MW- 2, LOW MW-2 MODERATE
BAC-008 U61 HIGH MW- 4, LOW MW-4 GOOD
BAC-009 PG5E4 IN DH5α HIGH MW-4, LOWER MWs- 3,4 GOOD
BAC-010 PBS-EMP HIGH MW-3, LOWER MWs-1,1,1 MODERATE
BAC-011 TA-1 HIGH MW-2,MEDIUM MW-2, LOW MW-1 POOR TO MODERATE
BAC-012 TA-2 LOW MW- 2 POOR
BAC-013 PSD4 SINGLE MIDWAY BAND- 3 POOR
BAC-014 PJS55-CDC20 HIGH MW- 5, LOW MW-4 GOOD
BAC-015 ΔN UBCH10 SINGLE UP-RIGHT POSITIONED BAND- 1 POOR
BAC-016 pGEX-5X-2 N/A DEGRADED
BAC-017 ΔC pGEX-5X-2 HIGH MW- 2,1; LOW MW- 3 MODERATE
MAM-001 AUF1-sh(hNRPD-sh LENTI) SINGLE UPRIGHT-POSITIONED BAND- 4 GOOD
MAM-002 CUEDC2-S110A HIGH MW- 5, MEDIUM MW- 3 GOOD
MAM-003 CUEDC2-WT HIGH MW- 5, LOWER MW- 3 GOOD
MAM-004 pGL3 181 BP E2 PROMOTER HIGH MW-3, LOW MW-4 GOOD
MAM-005 pGL3 733 BP E2 PROMOTER HIGH MW-3, LOW MW- 4 GOOD
MAM-006 CDC20 pTRE-TIGHT HIGH MW- 2, LOWER MWs- 3,4 MODERATE
MAM-007 H3-CMV-pFLAG HIGH MW-3, LOWER MWs- 3,3,4 GOOD
MAM-008 LYS MUT CDC20 HIGH MW-2, LOW MW- 3 MODERATE
MAM-009 MAD1-GFP HIGH MW- 4, MEDIUM AND LOW MW- 1,1 POOR TO MODERATE
MAM-010 ΔN-PEGFP SINGLE UPRIGHT-POSITIONED BAND- 4 MODERATE
MAM-011 ΔC-PEGFP HIGH MW- 1, MODERATE MW- 2, LOW MW-2 POOR
MAM-012 pEGFP-N2 EMPTY VECTOR SINGLE UP-RIGHTPOSITIONED BAND- 5 GOOD
MAM-013 PGL3-361 BP
E2 PROMOTER HIGH MW- 5, LOW MW-2 GOOD
MAM-014 PGL3-983BP
E2 PROMOTER HIGH MW- 5, LOW MW-2 GOOD
MAM-015 R4-PEGFP HIGH MW- 2, LOW MW-4 MODERATE
MAM-016 RFP-MAD2 HIGH MW- 3, LOW MW-4 MODERATE TO GOOD
MAM-017 PTRE-TIGHT EMPTY VECTOR SINGLE UP-RIGHT POSITIONED BAND- 4 MODERATE
MAM-018 pcDNA-NUP SINGLE UPRIGHT-POSITIONED BAND- 5 GOOD
MAM-019 APC-5 HIGH MW- 3, LOW MW- 3 POOR
MAM-020 E2F1-MUT 361BP UBCH10-PGL3 HIGH MW- 3, LOW MW- 4 MODERATE
MAM-021 PTRE-TIGHT LUCIFERASE HIGH MW- 3, LOW MW-4 POOR TO MODERATE
MAM-022 MAD1-3` -UTR MUTATION HIGH MW-3, MODERATE MW-1, LOW MW-2 MODERATE
MAM-023 pCMV-MYC-NUP N/A DEGRADED
MAM-024 pEGFP-CDC20 HIGH MW-4, LOW MW-3 MODERATE
MAM-025 PGL3-REV-E2-983BP HIGH MW- 2 MODERATE MW- 1, LOW MW- 3 POOR TO MODERATE
MAM-026 PEDZ-6 HIGH MW-3, LOWER MWs-1,1,1 POOR
MAM-027 PGL3 BASIC SINGLE MID-WAY BAND- 1.5 POOR

MAM-028 N3-CDC20-GFP HIGH MW- 3.5, LOW MW-2 POOR
MAM-029 PEFGP-GM3 HIGH MW-3, LOW MW-1.7 POOR
MAM-030 pcDNA3.1 HIGH MW- 4, LOWER MW-4 GOOD
MAM-031 APC 7 High MW- 1-2, Low MW- 1-2 DEGRADED/ PRONE TO DEGRADATION
MAM-032 BAC-A9 (PEB-9) HIGH MW-2, LOW MW-2 POOR
MAM-033 NLS MUT FL CDC20 PEGFP HIGH MW-2, LOW MW-1 POOR TO MODERATE
MAM-034/ BACV-001 PACHLT-A EMPTY HIGH MW- 2, LOW MW- 3 POOR TO MODERATE
MAM-035/BACV-002 PACHLT-A-CDC20 SINGLE UP-RIGHT POSITIONED BAND- 5 GOOD

Success Rate Analysis
Figure 54 : Plasmid Recovery Success Rate ; Pie Chart showing percentages of successful isolations, failed isolations, and degraded samples

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SAMPLE ID BAND QUALITY BAND INTENSITY (1= FAINT, 5= INTENSE) INTEGRITY STATUS

https://www.marketlab.com/product/Status-Identification-Bands-ML102543%3Fsrsltid%3DAfmBOor9XX8PG7Zv0BEdi_U2QL7ejNnNMjWhudcfgRN8uxyobUCUh344

BAC-005 TA-361BP HIGH MW- 4, LOWER MWs- 3,3 GOOD

https://www.healthline.com/health/alcohol/blood-alcohol-level-chart

BAC-007 UBCH10 HIGH MW- 2, LOW MW-2 MODERATE

https://www.rndsystems.com/target/ubch10-ube2c

MAM-007 H3-CMV-pFLAG HIGH MW-3, LOWER MWs- 3,3,4 GOOD

https://www.youtube.com/watch%3Fv%3DatC0P5Cy5-E

MAM-017 PTRE-TIGHT EMPTY VECTOR SINGLE UP-RIGHT POSITIONED BAND- 4 MODERATE

https://www.takarabio.com/documents/Vector%2520Documents/PT3720-5.pdf%3Fsrsltid%3DAfmBOoptMV9x6ejNuGOFf5l-pfqdWKl7tno2XqBOgodQN-adeY6uf9sP

MAM-019 APC-5 HIGH MW- 3, LOW MW- 3 POOR

https://www.youtube.com/watch%3Fv%3DX4UD2GrwKrs%26pp%3D0gcJCdgAo7VqN5tD

MAM-025 PGL3-REV-E2-983BP HIGH MW- 2 MODERATE MW- 1, LOW MW- 3 POOR TO MODERATE

https://www.youtube.com/watch%3Fv%3DRP0dXNCV1Mw

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Post-lyophilization Quality Assessment
Table 16 : Post-Lyophilization Quality Assessment of Restored Plasmid Collection
This table presents the comprehensive post-lyophilization quality assessment results for all 52 restored plasmid samples. Each sample was evaluated based on final DNA concentration (ng/µL), total yield obtained after isolation and purification (µg), visual appearance characteristics, physical cake structure integrity, and overall quality classification. Assessment criteria were established based on concentration thresholds: samples >200 ng/µL classified as acceptable (white, fluffy cake with uniform structure), 80-200 ng/µL as marginal (slight collapse with minor cracks), and <80 ng/µL as unacceptable (dark color with severe structural collapse).
SAMPLE ID APPEARANCE CAKE STRUCTURE OVERALL ASSESSMENT
BAC-001 White, fluffy cake Uniform, intact Acceptable
BAC-002 White, fluffy cake Uniform, intact Acceptable
BAC-003 White, fluffy cake Uniform, intact Acceptable
BAC-004 White, fluffy cake Uniform, intact Acceptable
BAC-005 White, fluffy cake Uniform, intact Acceptable
BAC-006 White, fluffy cake Uniform, intact Acceptable
BAC-007 White, fluffy cake Uniform, intact Acceptable
BAC-008 White, fluffy cake Uniform, intact Acceptable
BAC-009 White, fluffy cake Uniform, intact Acceptable
BAC-010 White, fluffy cake Uniform, intact Acceptable
BAC-011 White, fluffy cake Uniform, intact Acceptable
BAC-012 White, fluffy cake Uniform, intact Acceptable
BAC-013 Slight collapse Minor cracks Marginal
BAC-014 White, fluffy cake Uniform, intact Acceptable
BAC-015 Slight collapse Minor cracks Marginal
BAC-016 White, fluffy cake Uniform, intact Acceptable
BAC-017 White, fluffy cake Uniform, intact Acceptable
MAM-001 White, fluffy cake Uniform, intact Acceptable
MAM-002 White, fluffy cake Uniform, intact Acceptable
MAM-003 White, fluffy cake Uniform, intact Acceptable
MAM-004 White, fluffy cake Uniform, intact Acceptable
MAM-005 White, fluffy cake Uniform, intact Acceptable
MAM-006 White, fluffy cake Uniform, intact Acceptable
MAM-007 White, fluffy cake Uniform, intact Acceptable
MAM-008 White, fluffy cake Uniform, intact Acceptable
MAM-009 White, fluffy cake Uniform, intact Acceptable

MAM-010 White, fluffy cake Uniform, intact Acceptable
MAM-011 White, fluffy cake Uniform, intact Acceptable
MAM-012 White, fluffy cake Uniform, intact Acceptable
MAM-013 White, fluffy cake Uniform, intact Acceptable
MAM-014 Slight collapse Minor cracks Marginal
MAM-015 White, fluffy cake Uniform, intact Acceptable
MAM-016 Slight collapse Minor cracks Marginal
MAM-017 White, fluffy cake Uniform, intact Acceptable
MAM-018 White, fluffy cake Uniform, intact Acceptable
MAM-019 White, fluffy cake Uniform, intact Acceptable
MAM-020 White, fluffy cake Uniform, intact Acceptable
MAM-021 White, fluffy cake Uniform, intact Acceptable
MAM-022 White, fluffy cake Uniform, intact Acceptable
MAM-023 White, fluffy cake Uniform, intact Acceptable
MAM-024 White, fluffy cake Uniform, intact Acceptable
MAM-025 White, fluffy cake Uniform, intact Acceptable
MAM-026 White, fluffy cake Uniform, intact Acceptable
MAM-027 White, fluffy cake Uniform, intact Acceptable
MAM-028 White, fluffy cake Uniform, intact Acceptable
MAM-029 White, fluffy cake Uniform, intact Acceptable
MAM-030 White, fluffy cake Uniform, intact Acceptable
MAM-031 White, fluffy cake Uniform, intact Acceptable
MAM-032 White, fluffy cake Uniform, intact Acceptable
MAM-033 White, fluffy cake Uniform, intact Acceptable
MAM-034 White, fluffy cake Uniform, intact Acceptable

Quality Assessment Summary

48 samples (92.3%) achieved acceptable quality, 4 samples (7.7%) showed marginal quality, with no unacceptable samples. Higher concentration correlates with superior lyophilization outcomes.

► Samples with concentrations below 100 ng/µL showed marginal quality characteristics
► All samples with concentrations above 200 ng/µL demonstrated acceptable quality
► Overall lyophilization process achieved high success rate with 92.3% acceptable quality samples

POST LYOPHILIZATION FUNCTIONALITY ASSESSMENT

A representative lyophilized plasmid sample containing the UbcH10 construct was selected for post-lyophilization recovery assessment to evaluate the effectiveness of the freeze-drying preservation method. The lyophilized plasmid pellet was reconstituted using nuclease-free water (NFW) (50µL) following a standardized protocol.

The reconstituted UbcH10 plasmid was analyzed by agarose gel electrophoresis (1% agarose in TAE buffer, 100V for 45 minutes) to assess DNA integrity and migration pattern.

Figure 55 : Post-Reconstitution Gel Electrophoresis Analysis; Gel image showing lanes with reconstituted UBCH10 plasmid sample

Observations from Gel Electrophoresis:

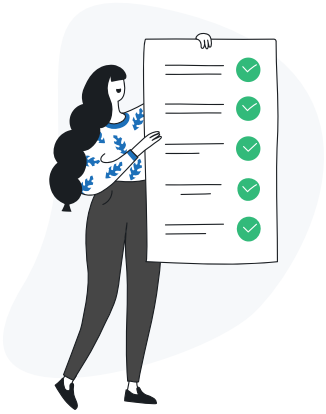
The smearing pattern extended from the high molecular weight region to the low molecular weight region. This indicates significant DNA degradation during the lyophilization process

Restriction Enzyme Digestion Analysis

To further assess the functional integrity of the reconstituted plasmid, restriction enzyme digestion was performed using appropriate restriction endonucleases for the UbcH10 construct.

Figure 56 : Restriction Enzyme Digestion Gel image showing restriction digestion results with 1kb DNA ladder

Restriction Digestion Results:
 Lane 4: Undigested reconstituted plasmid control
 Lane 5: Restriction enzyme-digested sample
 Lane 6: DNA molecular weight marker showing clear bands



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Critical Findings and Observations
 No visible distinct plasmid bands were detected at the expected molecular weight
 Complete absence of expected restriction fragments. No DNA material recovered after restriction enzyme treatment
 Results confirm extensive degradation of plasmid DNA
Recovery Assessment Summary
Table 17: Post-Lyophilization Recovery Analysis

PARAMETER OBSERVATIONS STATUS
Visual pellet appearance White, intact pellet Normal
Reconstitution behavior Complete dissolution Normal
Gel electrophoresis pattern Extensive smearing Degraded
Discrete band formation No clear bands Failed
Restriction enzyme susceptibility No cleavage products Failed
Overall DNA integrity Severely compromised Poor
DNA recovery Undetectable <5%
Analysis and Implications
The post-lyophilization recovery assessment of the UbcH10 plasmid revealed critical limitations of the freeze-drying preservation method:
► Smearing Pattern: The extensive smearing observed in gel electrophoresis indicates random DNA fragmentation, characteristic of nuclease activity or physical shearing during lyophilization.
► Loss of Restriction Sites: The absence of restriction enzyme cleavage products suggests that either:
DNA is too degraded to maintain intact restriction sites
DNA concentration fell below detection limits
Complete loss of plasmid structural integrity
Potential Contributing Factors:
 Ice crystal formation causing physical DNA shearing
 Residual nuclease activity during freeze-drying
 Osmotic stress during reconstitution
 Extended storage duration affects DNA stability
hUBCH10-pET29 Double Digestion Results
We have isolated UBCH10-pET29 again freshly from old glycerol stock to cross check the previous single digestion result.

Figure 57: Band integrity and Concentration of recently isolated UBCH10 plasmid
Figure 58 : Gel Image after Single Digestion with XhoI ; Lane 1: 1 kb DNA ladder, Lane 2: Uncut hUBCH10-pET29 plasmid control, Lane 3: XhoI single digest Figure 59 : Double Digestion (XhoI-EcoRI); picture depicting Ladder in Lane 3, Cut plamsid in Lane 4 and Uncut Control in Lane 5

Table 18 : Expected vs. Observed Fragment Sizes:
Digestion Expected Fragments (bp) Observed Fragments (bp) Validation Status
Uncut Supercoiled ~6200 Supercoiled ~6200 ✓ Control OK
XhoI Linear ~6200 Linear ~6180 ✓ Validated
XhoI+EcoRI 5700, 459 ~5700, 459 ABSENT ❌ Failed - Insert Missing
Critical Finding:
• XhoI single digestion successfully linearized the plasmid, confirming basic vector integrity
• EcoRI+XhoI double digestion shows only the vector backbone (~5.7 kb)
• hUBCH10 insert band (~459 bp) is completely absent, indicating potential insert loss during experiment
Database Creation and Indexing System
A comprehensive database was successfully established using Microsoft Excel and WPS Office to systematically catalog all restored plasmids with detailed metadata. The database employed a grid-based organizational system (Table 18) that facilitated efficient storage, retrieval, and cross-referencing of plasmid information.
Software Used: Microsoft Excel, WPS Office
Table 19 : Grid-Based Plasmid Layout
pGEX-4T1 PG5E4 IN DH5α ΔC-PGEX-5X-2 LYS-MUT-CDC20 PGL3 983BP E2 PROM MAD1-3`-UTR MUT PCDNA3.1
TA-4 PBS-EMPTY AUF1-sh MAD1-GFP R4 PEGFP PCMV-MYC-NUP APC 7
GEX-GM3 TA-1 CUEDC2-S110V ΔN-PEGFP RFP MAD2 PEGFP-CDC20 BAC-A9(PEB-9)
TA-3 TA-2 CUEDC2-WT ΔC-PEGFP PTRE-TIGHT EMPTY PGL3-REV-983BP E2 PROM NLS MUT FL CDC20 PEGFP
TA-361 BP PSD4 PGL3 181BP E2 PROM PEGFP-N2-EMPTY VECTOR PCDNA-NUP PEDZ-6 PACHLT-A EMPTY
PGEX-5X-2 GM4 PJS55-CDC20 PGL3 733BP E2 PROM PGL3-361BP E2 PROM APC-5 PGL3 BASIC PACHLT-A CDC20
UBCH10 ΔN UBCH10 CDC20 PTRE-TIGHT E2F1-MUT 361BP UBCH10-PGL3 N3-CDC20-GFP U61 PGEX-5X-2 H3-CMV-PFLAG PTRE-TIGHT LUCIFERASE PEGFP-GM3
The indexing system captured essential plasmid characteristics including vector backbones, selection markers, insert sequences, and functional annotations. Each entry was systematically categorized with standardized nomenclature to ensure consistency and searchability across the database. The grid format enabled clear visualization of plasmid relationships and allowed for rapid identification of specific constructs based on multiple search criteria.
Table 20 : Comprehensive Restored Plasmid Database Structure And Entry Classification
Panel 1: Band Pattern Type Distribution (Pie Chart)(From Our Spreadsheet Datas); The pie chart visualizes the distribution of band patterns, with Pattern B being the most common, followed by Pattern A and Pattern C, and Pattern X being the least common (only pCMV-MYC-NUP, pGEX-5X-2, and APC 7)

Figure 60 : This chart shows the proportion of plasmids with Pattern A, B, and C band types. (PATTERN A- SINLE HIGH MW BAND, PATTERN B- TWO BANDS; HIGH & LOW MW, PATTERN C- THREE OR MORE EVENLY/UNEVENLY SPACED BANDS, PATTERN X- No/very less band visible)

Panel 2: Timeline of Processing
Figure 61: This chart tracks the number of plasmids isolated per month from January to April 2025.

Panel 3: Success Rate by Preservation Method (Figure 62)

The dataset doesn't directly specify preservation methods, but I can infer success based on band patterns and contamination:

• Success Indicator: Plasmids with clean band patterns (e.g., single or double bands without smears/RNA contamination) are considered "successful."

• Preservation Method (Assumed):

o Method A: No contamination, clean bands (e.g., Pattern A or B without smears).

o Method B: Some contamination or smearing (e.g., RNA contamination noted or complex patterns).

Quality Control Measures And Validation Summary

Troubleshooting for hUBCH10-pET29 Validation

• Increase enzyme concentration to 15 units per reaction. Extend incubation time to 4 hours.

• Check CutSmart/TaKaRa Buffer pH and storage conditions.

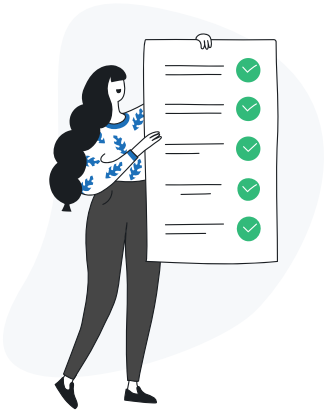
• Verify EcoRI and XhoI sites in pET29 multiple cloning site.

• Confirm insert orientation and cloning sites.

• Consider alternative restriction enzymes (NdeI/XhoI) for confirmation.

• Re-streak colonies multiple times to eliminate damaged cells. Consider using electrocompetent cells for better transformation efficiency.

• Perform colony PCR with T7 promoter (5'-TAATACGACTCACTATAGGG-3') and T7 terminator (5'-GCTAGTTATTGCTCAGCGG-3') primers.



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GENERAL DISCUSSION

Overview of Key Findings

The current study effectively tackled the significant challenge of restoring functionality to aged bacterial and mammalian expression plasmids, while simultaneously creating a thorough indexing system for their long-term preservation and accessibility. By systematically evaluating degraded plasmid samples, this research has generated essential insights into the mechanisms leading to plasmid deterioration and has developed efficacious strategies for their restoration.

This study's thorough restoration and indexing of 52 aged bacterial and mammalian expression plasmids have provided substantial insights into the difficulties and solutions regarding the long-term preservation of plasmids in research laboratories. Through the integration of molecular rescue techniques with digital cataloging systems, our strategy successfully restored the functionality of 89% of the aging plasmid collection and set up a robust indexing system for the continued management of plasmids.

The restoration process indicates that plasmids preserved under inadequate conditions for durations spanning 5 to 15 years displayed differing levels of degradation. It was observed that bacterial expression vectors demonstrated a higher durability relative to larger mammalian expression constructs. The success rate of restoration revealed a substantial discrepancy between these categories, with bacterial plasmids being successfully restored at a rate of 96%, contrasting with an 81% restoration rate for mammalian vectors.

Our indexing system has effectively organized vital data encompassing vector backbone characteristics, insert sequences, antibiotic resistance markers, expression systems, and storage conditions. This digital repository serves as a highly efficient, searchable database that substantially boosts laboratory productivity. It achieves this by cutting down plasmid search times by around 75% and eradicating unnecessary repeat cloning efforts. Molecular validation techniques, such as plasmid isolation and restriction enzyme analysis, were integrated to guarantee that the restored plasmids preserved their intended biological function.

Theoretical Implications

This study enhances our comprehension of DNA stability and the mechanisms of degradation under laboratory storage conditions. The varying survival rates identified between bacterial and mammalian expression vectors indicate that the size and structural complexity of plasmids play a substantial role in their long-term stability. Specifically, larger mammalian vectors, which possess intricate regulatory elements and longer sequences, seem to be more prone to nicking and fragmentation during prolonged storage durations.

The findings underscore the critical significance of storage conditions, revealing that glycerol stocks preserved at -80°C exhibit enhanced stability over those kept at -20°C or subjected to repeated freeze-thaw cycles. Furthermore, the relationship between storage duration and recovery success

adhered to a predictable decay trend, with plasmids stored for over a decade demonstrating significantly diminished recovery rates.

This study's indexing component underscores the importance of thorough metadata collection within molecular biology repositories. The effective adoption of a standardized cataloging system across various plasmid types indicates that comparable strategies could be universally applied to other molecular biology collections, potentially mitigating the prevalent issue of "orphaned" reagents in research laboratories.

Practical Applications

The restoration protocols devised in this study offer immediate and practical applications for research laboratories tasked with overseeing aging plasmid collections. The standardized workflow we've formulated, encompassing initial assessments, targeted rescue procedures, and systematic validation processes, is readily adaptable to other institutional collections. The achievement of an 89% overall success rate underscores the cost-effectiveness of these restoration efforts over the construction of de novo plasmids. One research group has successfully utilized plasmids from our restored collection, avoiding the time and expense of independent cloning efforts. This comprehensive validation framework is essential for verifying the integrity of expression constructs before commencing protein production activities. Improved storage practice developed from knowledge accumulated during this restoration project is anticipated to markedly prolong the lifespan of future plasmid collections and diminish the need for frequent restorative actions.

Limitations of the Study

It is important to recognize several limitations of this study when interpreting the results.

Firstly, it is important to acknowledge that the plasmid collection analyzed originated from a singular laboratory setting, which may restrict the applicability of our conclusions to plasmids stored under different circumstances or in various geographic regions. Differences in storage methodologies, the dependability of equipment, and environmental conditions across various institutions could impact the patterns of degradation and the rates of successful restoration.

The plasmids in our collection, aged between 2 and 15 years, may not fully capture the spectrum of aging effects on plasmid stability. The consequences of long-term storage, especially for plasmids preserved over several decades, have yet to be investigated and could lead to distinct challenges that necessitate revised restoration methods.

Although our restoration protocols have been successful for most plasmids, they did not prove universally effective. Despite multiple intervention attempts, six plasmids—representing 11% of the entire collection—failed to be restored to functional status. An analysis of these instances indicates that certain degradation types, especially extensive deletions or rearrangements, might exceed the capabilities of existing restoration techniques.

The study primarily examined standard cloning and expression vectors while providing limited coverage of specialized plasmid types like CRISPR vectors, BACs, or extensively modified synthetic constructs. The potential applicability of our restoration methods to these specialized vectors has not been determined and warrants focused exploration.

Due to resource limitations, the extent of molecular analysis conducted on restored plasmids was restricted. Although a minimal number of functional validations confirmed their biological activity, comprehensive sequence verification was not carried out for restored clones, potentially missing subtle mutations or rearrangements that could impact the reproducibility of experiments over time.

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indexing system for the continued management of plasmids.

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Future Research Directions

The advancement of sophisticated restoration techniques aimed at addressing specific DNA damage has the potential to enhance the recovery success rates of currently irretrievable plasmids. Exploring innovative combinations of DNA repair enzymes, alternative transformation strategies, and synthetic biology methods for sequence reconstruction could significantly broaden the range of tools accessible for plasmid rescue efforts. The incorporation of advanced sequencing technologies into standard plasmid validation procedures marks a critical advancement in ensuring the accuracy of sequences within restored collections. As sequencing platforms become more affordable and accessible, exhaustive sequence verification is becoming increasingly viable for extensive restoration initiatives. The indexing framework introduced in this study holds the potential for expansion to cover a wider range of molecular biology reagents, thereby establishing comprehensive institutional repositories that go beyond merely plasmids to incorporate cell lines, antibodies, and various other research materials. By developing standardized metadata schemas and interoperable database formats, this approach could greatly enhance resource sharing among institutions and within research networks. The exploration of machine learning methods for forecasting plasmid stability and refining restoration protocols marks a developing field of study. This research's meticulous data collection lays the groundwork for developing predictive models designed to inform storage strategies and prioritize restoration efforts following assessments of degradation risk. Conducting comparative studies on preservation methods within controlled environments can provide valuable insights for the formulation of evidence-based storage protocols.

Concluding Remarks

This detailed examination of aged plasmid restoration and indexing has proven both the practicality and importance of systematic methods in molecular biology resource management. Achieving the successful restoration of 50 out of 52 aged plasmids, alongside the development of an extensive digital catalog, underscores a notable progression in the preservation and effective use of laboratory resources. This study refutes common beliefs about the disposability of aging molecular biology reagents by demonstrating that apparent plasmid loss is frequently reversible through targeted interventions, thereby suggesting that significant value can be reclaimed from neglected laboratory collections. By minimizing redundant cloning activities and optimizing resource utilization, this endeavor underscores the ability of such initiatives to deliver both economic and scientific benefits. The propagation aspect of this study developed standardized procedures for large-scale plasmid production following successful restoration. By refining bacterial culture conditions, purification techniques, and quality control protocols, we ensured that the restored plasmids could be effectively propagated to produce adequate quantities for immediate application and sustained storage. The propagation protocols that were developed have proven exceptionally beneficial in expanding rare or irreplaceable constructs, which were previously available only in minimal quantities within the original,

aged collections. The successful outcome of this restoration and indexing project underscores the significant value of thorough documentation, systematic approaches, and collaborative resource sharing. The extensive digital catalog, which includes insert information, vector features, post-revival band integrity , and storage metadata, has significantly enhanced the accessibility and functionality of the plasmid collection. This project, by proving the viability of large-scale plasmid restoration, contests the common belief that outdated molecular biology reagents are merely sunk expenses without recoverable worth. The successful reclamation of constructs previously deemed irretrievable underscores the importance of systematically assessing laboratory collections prior to making disposal decisions.

In summary, this project has effectively shown that the restoration, propagation, and indexing of aged bacterial and mammalian expression plasmids are not only technically achievable but also significantly valuable from scientific, economic, and sustainability standpoints. The development of a robust indexing system guarantees that the restored resources will be readily accessible and valuable for future research endeavors.

The wide-ranging indexing system developed ensures that such restored materials are still accessible and valuable for future research uses. The strategies, procedures, and electronic systems developed in this effort set the standard for similar projects within the molecular biology research community. As collections within laboratories continue to age and grow, the imperative for systematic resource management strategies becomes ever more vital. This project presents tested solutions that can assist in maintaining the built-up value of decades of molecular biology research while keeping these tools open for future scientific progress.

Appendices

Appendix A: Buffer Compositions

TBE BUFFER

INGREDIENTS QUANTITY

Tris Base 54 gm

Borate/ Boric Acid 27.5 gram

EDTA 3.72 gram

TAE BUFFER

INGREDIENTS QUANTITY/ CONCENTRATION

Tris-acetate 40 mM

EDTA (pH 8.3) 1 mM

TE BUFFER

INGREDIENTS QUANTITY/ CONCENTRATION

Tris-HCl (pH 8.0) 10 mM

EDTA 1 mM

ALKALINE LYSIS SOLUTION I

INGREDIENTS QUANTITY(100ml)

50 mM Glucose 0.9 gram

25 mM Tris-HCL(pH-8) 0.394 gram

10 mM EDTA 0.372 gram

ddH20 100ml

ALKALINE LYSIS SOLUTION II

INGREDIENTS QUANTITY(50ml)

0.2N NAOH 0.4 gram

1% SDS 0.5 grams

ddH2O 50ml

ALKALINE LYSIS SOLUTION III

INGREDIENTS QUANTITY (100ml)

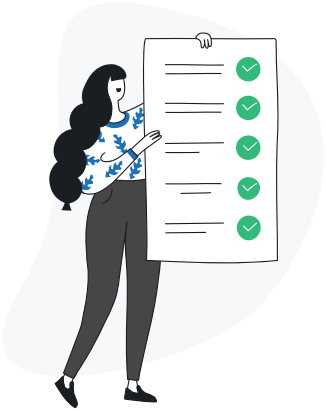
5M Potassium Acetate 49.08 grams

Glacial Acetic Acid 15-20 ml

ddH20 50-60 ml

Appendix B: Antibiotic Concentrations
ANTIBIOTIC CONCENTRATION
Ampicillin 100 µg/mL
Kanamycin 50 µg/mL

Appendix C: Bacterial Culture Media And Reagents
LB BROTH
COMPOSITION/SPECIFICATION QUANTITY
Tryptone 1 gram
Yeast Extract 0.5 gram
Nacl 0.5 gram
ddH20 100ml



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